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THE EFFECT OF FEEDING MAMMALIAN THYROID POWDER ON GROWTH RATES OF IMMATURE GUPPIES[1]

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Received June 20, 1960

Attempts to influence the growth of young fish through the use of mammalian thyroid substances has given both positive (Gerbelsky and Saks, 1947; Hopper, 1952) and negative (Grobstein and Bellamy, 1939; Smith and Everett, 1943) results. In the case of the positive results, both groups raised the young fish in solutions of the hormones, thyroxin in the former and mammalian thyroid powder in the latter instances. In the case of the negative results, the hormones employed were the same, but administration was by feeding. The fact that opposite effects were obtained using the same species, *Lebistes reticulatus*, and the same hormone, mammalian thyroid powder, indicated that the different results might be explained on the basis of the different methods of administration. Since the author has reported previously that immature guppies raised in a 0.01% solution of mammalian thyroid powder show a significant increase in growth and time of gonopod differentiation (Hopper, 1952) and also an increase in oxygen consumption (Hopper, 1959), the present experiment was conducted to determine if, under the same experimental conditions, the effect of the thyroid powder on growth and differentiation could be reproduced by feeding rather than by raising the fish in solutions of the thyroid powder.

MATERIALS AND METHODS

In each experiment growth rates were compared between offspring of a single female. The young were divided into 2 groups of which one was fed 50 milligrams of mammalian thyroid powder daily with the food while the other served as a control. In about half of the

[1] Supported by a grant from the National Science Foundation.

experiments the fish were raised in 3000 ml. tanks in which the water was changed semiweekly in both controls and experimentals. In the other half the fish were kept in 3000 ml. tanks planted with *Cobomba* and *Sagittaria* and the water was not changed during the course of the experiment. The former conditions were imposed in order to have some basis of comparison with previous experiments where fish were raised in solutions of the hormone and the water had to be changed semiweekly in order to prevent contamination.

Biweekly measurements of length from the front of the upper lip to the base of the caudal fin were made using an ocular micrometer. Over 200 fish, the offspring of 20 females, were used in the experiments.

Results

The results of these experiments are presented in Table 1. Reference to this table shows that feeding mammalian thyroid powder had no effect on length increases at any time in the experiment. Control fish in the group without plants averaged slightly larger than thyroid-fed fish at each time interval, but the differences were not significant. Thyroid-fed fish in the group raised with plants in the aquaria averaged slightly larger at 20 and 40 days than controls and slightly smaller at 60 days of age, but again the differences were not significant.

TABLE I

Length in Mm. of Thyroid-treated and Control Fish

Age in days	Without Plants		With Plants	
	Control	Thyroid	Control	Thyroid
20	7.35 ± .55	7.22 ± .47	8.17 ± .62	8.62 ± 1.16
40	8.05 ± .95	7.72 ± .76	9.62 ± 1.06	9.69 ± 1.13
60	8.55 ± 1.08	8.46 ± 1.24	10.79 ± 1.11	10.58 ± 2.2

However, comparison of controls in tanks where the water was changed with those in tanks where the water was not shows a significant increase in length in fish in the tanks with plants in each time period compared. The percent difference in length was 11% at 20 days, 19% at 40 days and 26% at 60 days. Thyroid-fed fish in tanks with plants also showed significant increases in length over thyroid-fed fish in tanks without plants, the percent differences in length being 20% at 20 days, 25% at 40 days and 26% at 60 days. Also, control fish in tanks with plants showed significant increases in length

over thyroid-fed fish in tanks without plants. In this case the differences were 13% at 20 days, 25% at 40 days and 28% at 60 days. Comparisons between fish in tanks with plants and those in tanks without plants strictly speaking should not be made since the experiments were not set up to make such comparisons; since the fish being compared were not the offspring of the same parents, nor were they raised under exactly the same conditions of light and temperature. However, the differences in growth rates between these groups are so large that in all probability experiments set up to make these comparisons would yield similar results.

Histological examination of the thyroid region in experimental and control fish showed no significant difference in differentiation of the follicles. The number of follicles, the height of the epithelium and the amounts of colloid were comparable throughout the course of development. Sections through the gonads showed that thyroid-feeding had no effect on the differentiation of the tests or ovaries. However, the fish raised in tanks with plants matured more rapidly than those raised in tanks without plants.

Discussion

These results, which show that feeding thyroid powder has no effect on growth, indicate that the method of administration of the hormone is important to the end results since previous experiments with the same species have demonstrated that thyroid powder can be effective in increasing growth rates of immature fish if the powder is mixed with the aquarium water (Hopper, 1952). The present results may serve to explain some of the previous negative reports (Grobstein and Bellamy, 1939; Etkin, Root and Mofshin, 1940; Smith and Everett, 1943; Smith, Sladek and Kellner, 1953) where the method of administration was by feeding. The only report which presents positive effect on growth by feeding is that of Nigrelli, Goldsmith and Charipper (1946) who used this method of administration effectively in counteracting the growth-inhibiting effects of thiourea on the guppy.

In addition, the more rapid growth of the fish in aquaria in which plants were present and under conditions where the fish were not disturbed, except when they were measured, indicates the importance of providing similar experimental conditions where results from dif-

ferent laboratories are to be compared. A preliminary report (Hopper, 1950) on the comparison of growth rates of control and thyroid-treated fish, used the method of mixing the powder with the water and therefore, did not provide plants and also involved changing the water twice a week. This experiment showed that the thyroid-treated fish grew considerably more rapidly than their controls. However, these results were criticized (Smith, Sladek and Kellner, 1953) on the grounds that in their laboratory they were able to bring control fish to sexual maturity in approximately the same time reported by this author using thyroid power. In this report (loc. cit.) there was no pretension that optimum conditions for growth were present, but rather we were using an experimental method which was necessary in order to give comparable results between controls and thyroid-treated fish.

The fact that increase in length is more rapid in fish raised in tanks with plants could be due to a number of factors. Presumably the fish could benefit not only from the "aging" of the water and the presence of various micro organisms, but also from the freedom from handling and disturbance during the semi-weekly water changes. Results in the present paper indicate that we might expect about a 20% increase in growth rate in fish raised in tanks with plants over those in tanks without plants. A previous paper (Hopper, 1952) reported a 40% increase in growth in fish raised in tanks in which thyroid powder was mixed with the water over control fish raised in tanks without plants, but in which the water was changed twice weekly. It would be interesting to compare growth rates of fish raised in solutions of thyroid powder with those of fish raised in tanks with plants.

Summary

1. Giving 50 mg. of mammalian thyroid powder daily with the food had no effect on increase in length over controls.

2. Fish raised in tanks without plants where the water was changed twice weekly did not grow as rapidly as those raised in tanks containing green plants in which the water was not changed.

3. It is concluded that the method of administration of the hormone, as well as the experimental procedure, produce markedly different effects which may well explain some of the controversial results appearing in the literature on the role of the thyroid gland in growth and differentiation in teleosts.

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EXPERIMENTS ON REGENERATING RAT DIGITS

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INTRODUCTION

Mammalian limbs are often considered to be incapable of regeneration. This contrasts with the situation in amphibians in which amputated limbs can be stimulated to regenerate by a variety of means, such as heat shock, NaCl treatments (Rose, 7, 8, 9), and surgical traumatization of tissue (Polezhayev, 4). Schotté (10) announced that there was some evidence concerning the possibility of stimulating regeneration of the digits of a mouse by a variety of means, but he did not detail this evidence. This paper describes the partial regeneration of the digits of the right forelimb of two-day old rats, the stimulation being the alternating application of trypsin and $CaCl_2$ solutions.

METHOD

The fourth and fifth digits were removed from the right mani of 20 Wistar rats two days after their birth. A surgical snare** (Fig. 1) with a spring-activated adjustable wire loop 0.1 mm. thick cut through

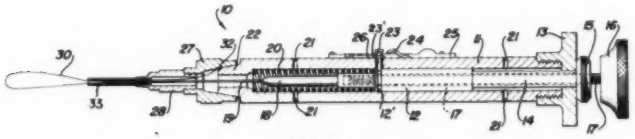

FIGURE 1

A surgical snare, Patent No. 2,856, 933. No. 30, wire, 0.1 mm. thick. No. 19 is a rod attached at one end of the wire while the other end of the rod has a screw nut making the loop adjustable. Spring, No. 20, is released by the button at No. 23. Other numbers, while important, are of no further importance to this paper. Figure is about ½ actual size.

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** The snare, Pat. No. 2,856,933, may be obtained from Herbert Hildebrand, Valley Circle Drive, Miller Place, Long Island, New York.

the digits as shown in Fig. 2; care was taken to amputate proximad to the point separating these digits.

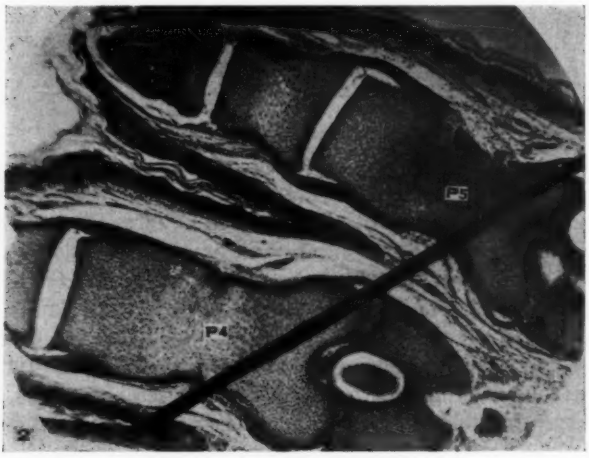


FIGURE 2

Frontal section of intact proximal phalanges of the fourth and fifth digits of a two-day-old rat at the time of operation. The black line shows the approximate course followed by the wire loop of the snare. P4 is the proximal phalanx of the 4th digit. P5 is the proximal phalanx of digit #5 of the right manus. Magnification about 35 ×.

The snare has several advantages that are not readily apparent. No trimming of the stump on the day following amputation is needed. Histological sections of the parts removed show how much tissue has been amputated. With methods that require trimming, more tissue is removed than can be histologically demonstrated. Damage to tissue is equal in all directions because all sides of the wire loop cut through the tissue simultaneously. The speed with which the wire loop is constricted insures a sharp cut, not only through soft tissue but through the cartilage as well. After the area to be cut has been looped with the wire of the snare, the operator pushes a button and further action of the tool is automatic. In these ways, the amputations performed in this experiment were standardized.

After amputation the 20 rats were divided into three groups, 12 in group A, 4 in group B, and 4 in group C. One day after extirpation the 12 rats in group A were treated at the site of amputation by alter-

nating applications of lyophilized crystalline trypsin with warmed 0.95% $CaCl_2$ solutions for a total of 35 to 40 minutes. A full round of treatment consisted of a three minute application of trypsin immediately followed by a three minute application of $CaCl_2$. After the scab material was completely removed—at the end of five or six continuous rounds of treatment—an additional continuous 5 to 10 minute application of $CaCl_2$ concluded the treatment.

Trypsin was applied by placing a drop of it on the amputation site, whereas the entire stump was dipped into the warmed $CaCl_2$ solution.

In group C, the 4 rats were treated in the same way as were group A animals, except that when the scab material became gelatinous, after 2 or 3 rounds of applications, treatment was terminated. Thus the scab material remained at the site of amputation. No treatment of any kind was given to the 4 rats in group B.

All litters were reduced to 4 per mother at the time of operation. It was found that in litters of 10 to 14 animals some experimental animals did not survive, nor gain weight as rapidly as when the litters were reduced in number. Mothers were given their daily food supply during the absence of the litters. Young were returned as a group to their respective cages near their nests, but not into their nests. After the mother rats returned their young to the nest area, additional nesting material was immediately made available. This procedure was repeated on the day of treatment. All of the 20 experimental animals survived.

Histological sections of the parts of the fourth and fifth digits removed at the time of amputation from all 20 animals mentioned above were fixed in 10% formaldehyde, sectioned at 12 microns, and stained with hematoylin and eosin. Sections from older rats were decalcified at the appropriate time.

Experimental Results

Gross Results

Group A rats developed projections (Fig. 3) at the fourth and fifth digital amputation sites in 9 out of 11 cases. Results from the twelfth animal were not valid. None of the group B or C controls developed projections (Fig. 4) at the sites.

FIGURE 3

Dorsal view of the right manus of A8, 38 days after treatment. Partially regenerated projections P4 and P5 are at the fourth and fifth digital areas, respectively. Magnification about 11 ×.

FIGURE 4

Dorsal view of the right manus of an untreated rat B1, 38 days after operation. There are no partially regenerated projections, P4 and P5, at the fourth and fifth digital areas. Distal direction is up. Magnification about 11 ×.

FIGURE 5

Longitudinal section of a reorganized proximal phalanx from a rat, A7, 98 days after treatment. New bone is above the black line. Distal end is up. Magnification about 35 ×.

FIGURE 6

The same section as Fig. 5, but a more distal view shows a triangular area of periosteal-type cell formation (B). Distally (up) scar material of the epidermis is not apparent. Magnification about 35 ×.

P4
P3
3
P4
P3
4
5
6

Tables 1 and 2 describe the relationship of treatment and projection development for each rat in groups A, B, and C from a gross point of view.

TABLE I

TIME-EVENT RELATIONSHIPS IN PARTIALLY REGENERATING AND NON-REGENERATING RAT DIGITS

Results (read notes in conjunction with table)

Experimental Animals	Days Scab Remains[1]	Days until complete healing[2]	Days after amputation yellow material noted[4]	Days yellow material remains	Day when projection first appeared
Group A					
A1	1	14	2	14	15
A2	1	9	2	9	12
A3	1	> 16[3]	2	> 16	14
A4	1	> 10	2	> 10	—[6]
A5	1	> 10	2	> 10	9
A6	1	> 10	2	> 10	9
A7 (Fig.)	1	> 10	2	> 10	9
Group B					
Controls					
B1 (Fig.)	9	9	4	5	—
B2	9	9	4	5	—
B3	9	9	4	5	—
B4	9	9	4	5	—
Group C					
Controls					
C1	9	9	[5]		—
C2	9	9			—
C3	9	9			—
C4	9	9			—

[1] The scab material in group A animals is removed 24 hours after amputation by the treatment described above. The scab material is removed by natural causes in groups B & C.

[2] Completed healing as used here means that state in which the tissue covering the amputation site is smooth; and nothing interrupts the smooth surface at a magnification of 1×.

[3] Healing was completed sometime between the date indicated and the next observation 8 days later.

[4] The nature of the yellow material is not known at this time. In the case of group A rats, this substance covers the entire amputation surface sometime between the end of treatment and the next observation period, one day later. In group B rats, the yellow material is located only at the outer edge of the scab, and is relatively minute in quantity.

[5] The blank spaces indicate that no observations were made concerning the yellow material in group C animals.

[6] The dash, —, indicates that no projections developed within the first 220 days following amputation. There were no further observations following this period.

Histological Results

Histological sectioning of 10 projections from 5 group A rats demonstrated that remodeling of the 10 cut proximal phalanges (Fig. 5) took place by 98 days following treatment. The reorganized

TABLE II

DAYS AFTER TREATMENT THAT PROJECTIONS ARE FIRST NOTED AT THE FOURTH AND FIFTH SITES OF AMPUTATION, AND MEASUREMENTS THAT WERE MADE OF THE LENGTHS (IN MM.) OF THESE NEW GROWTHS

Days after treatment	0			5			10			16			23		
	5[a]	4[b]	3[c]	5	4	3	5	4	3	5	4	3	5	4	3
Treated Group A Rats															
A8	0	0	—	0	1*	4	1*	2	5	3	3	7	4	4	9
A9	0	0	—	0	0	4	0	1*	5	2*	3	7	4	4	9
A10	0	0	—	0	0	5	—	—	—	0	0	6	3*	3*	9
A11	0	0	—	0	0	4	—	—	—	0	0	7	0	0	7

[a] Fifth projection in mm.
[b] Fourth projection in mm.
[c] Third digit, control, in mm.

Measurements of the lengths of the intact third digits were made from the same paw associated with amputated fourth and fifth digits at the times indicated. The measurements of the third digits acted as controls for the other measurement. All measurements are in terms of millimeters. The degree of error is ± 0.5 mm. Table II shows that the first appearance of projections is highly variable in terms of time. The animals in this chart are part of the group A treated animals. They are different individuals than appear in Table I. The symbol * is the first day that a projection is noted. The symbol —, means that no measurements were made at that time. Day 0 is the day of treatment. Observations were made immediately after completion of treatment.

Although no further measurements of the above nature were made after 23 days, the relative proportions of the reorganized digits to the control digit appeared to be the same at the end of the following 120 days.

parts of the phalanges had relatively narrow shafts and bulbous heads at the distal ends. The lengths of the entire reorganized phalanges were comparable to the lengths of uncut proximal phalanges. While there was no bone marrow formation in the reorganized parts of four of the cut phalanges, it was quite apparent in the reorganized parts of the six others.

A more distal area of the phalanx shown in Figure 5 (Fig. 6) shows a triangular area of periosteal-type cells beyond the distal end of the reorganized phalanx. These cells have not modeled a new phalanx. Figure 7 shows that about 59% of the total length of original tissue of the same cut phalanx from the rat of figures 5 and 6 was amputated one day after birth.

FIGURE 7

Frontal section of the part of the proximal phalanx (A) amputated with the rest of the fourth digit from the same animal as in Figure 5, 2 days after birth. Distal direction is up. Magnification about 35 ×.

FIGURE 8

Frontal section of an unorganized fourth and fifth proximal phalanx from an untreated rat 115 days old. Anastomosis (C) of these phalanges is in process. The fifth metacarpal (D) is articulating with the unorganized fourth proximal phalanx (E). F is the fifth cut phalanx. These cut phalanges are about ½ the length of reorganized phalanges. Magnification about 35 ×.

FIGURE 9

Frontal section of the right manus of a 3-day-old rat one day after treatment. Amputation area shown by arrows. P4 is part of the 4th cut phalanx. P5 is part of the fifth cut phalanx. Distal direction is up. Magnification about 15 ×.

FIGURE 10

Frontal section of the right manus of a 3-day-old rat 2 days after amputation. Amputation surface shown by arrows. Part of fourth cut phalanx, P4. Part of fifth cut phalanx, P5. Distal direction is to the right. Magnification about 15 ×.

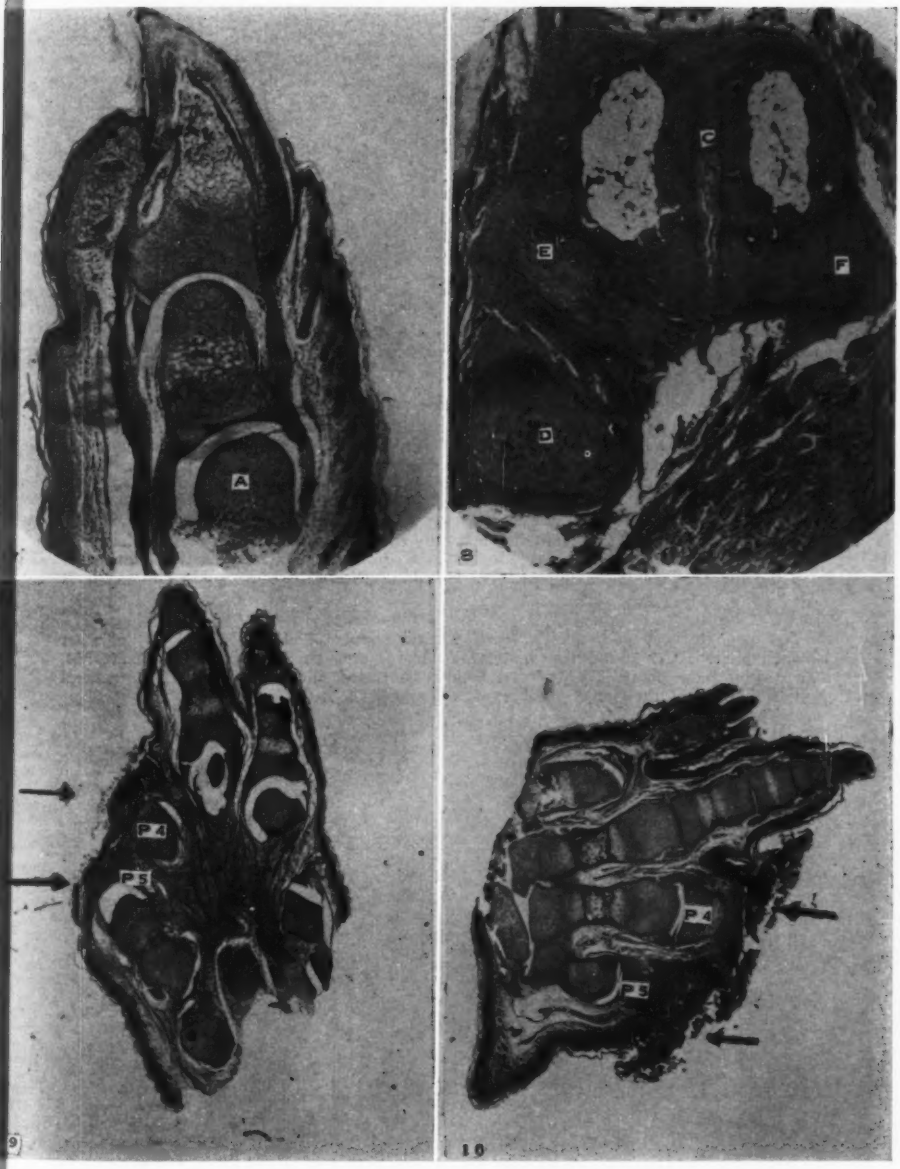

A
C
E
F
D
8
P4
P5
9
P4
P5
10

FIGURE 11

Frontal section of the right manus of a rat 8 days after treatment. Yellow exudate at surface (G). Part of fourth cut phalanx, P4. Part of fifth cut phalanx, P5. Distal direction is up. The arrow points to the channel. Magnification about 10 ×.

FIGURE 12

Frontal section of the right manus of a rat 15 days after treatment. H is the third intact digit. P4 and P5 are the reorganizing fourth and fifth proximal phalanges respectively. Note the remodeling processes taking place in the fourth cut phalanx. Distal direction is up. Magnification about 9½ ×.

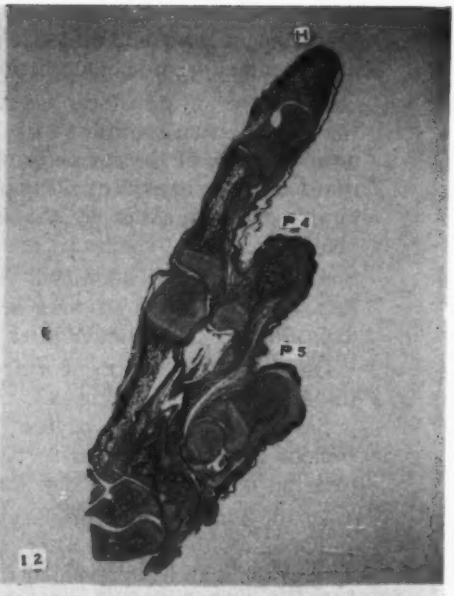
FIGURE 13

Frontal section of the amputated fourth and fifth digits from the same rat shown in Figure 12. The rat was one day old. P4 and P5 are the cut proximal phalanges of the fourth and fifth digits respectively. Distal direction is up. Magnification is about 15 ×.

FIGURE 14

X-ray photograph of the left manus of a rat about 100 days old. D2 is the intact second digit. P3, P4, and P5 are partially regenerated proximal phalanges. M3, M4, and M5 are metacarpals of normal length associated with their respective proximal phalanges. Distal direction is up. Magnification about 5 ×.

11



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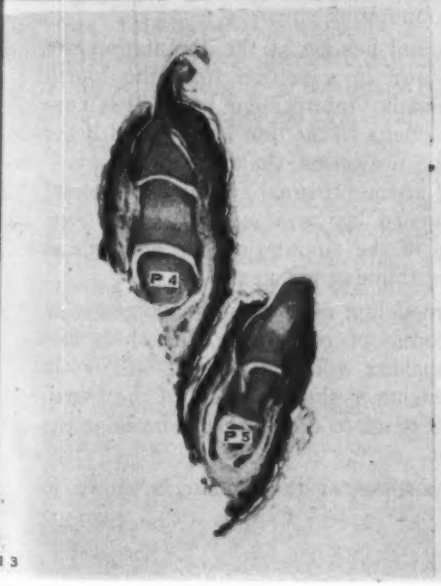

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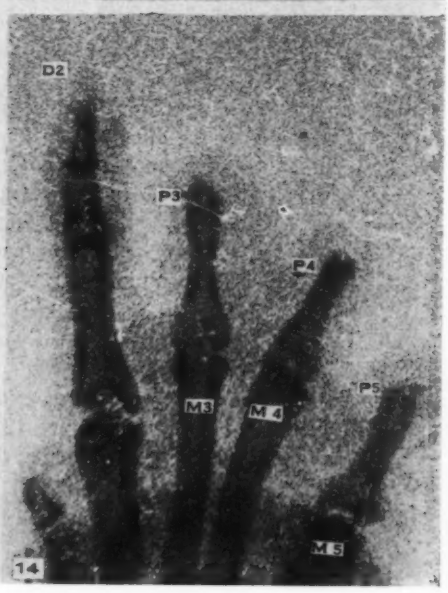

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Histological sections of 2 cut phalanges from one rat of group B did not differ from similar sections of 2 cut phalanges from one rat in group C.

Figure 8, from control rat B1, 115 days after operation, shows that no remodeling of the cut phalanges has developed, though bone marrow formation is evident. Cut phalanges are shown to have anastomized, and are about ½ the length of corresponding reorganized phalanges.

A group of four additional rats, three treated like the group A rats and one untreated were sectioned at their amputation sites 2 days, 9 days, and 16 days after operation.

One day after treatment a dry flaky scab is no longer present at the amputation site (Fig. 9). The wound exudate contains mostly polymorphonuclear leucocytes. The intercellular material is continuous in the stump matrix. None of the leucocytes or intercellular material is frozen into a dry scab.

In the control rat, a primary fibrin clot has formed at the amputation site within 2 days after operation (Fig. 10). The flaky structure of the scab has prevented free movement of the cellular material in the wound exudate about the amputation site.

Eight days after treatment, wound healing at the amputation site is not complete (Fig. 11) as shown in a section from the fourth phalanx of one of the treated animals. Intercellular material is continuous from the cut end of the phalanx to the thin layer of epithelial cells at the outermost edge of the projection via a narrow channel. Mesenchymal cells are condensed at the proximal end of this channel both about and within it. Thickened layers of epithelial cells surround the channel at the surface of the amputation site and extend over the entire wound area before thinning out again.

Fifteen days after treatment modeling of the cut phalanges is in progress. (Fig. 12). In the process of remodeling, the shaft and distal head of the reorganized phalanx will thin out but will retain their shape. Cells from the periosteum at the distal end of the fourth remodeled phalanx in Figure 12 extend to the epithelial layer at the distal end of the projection.

Figure 12 shows amputated portions of the phalanges shown in

Results From One Rat Treated In Utero

In the light of the above results, it seems proper to mention an animal not included in the above groups whose results were similar in nature to those already reported upon, although the method was somewhat different. Three days before the birth of this rat, the third, fourth, and fifth digits of the left manus were amputated with a surgical snare (Fig. 1), immediately after which ½ cc. of a solution of Nile Blue Sulphate in a 0.95% Tyrode solution (1 part to 200,000 parts respectively) was injected into the amniotic sac of the rat. Treatments of $CaCl_2$ were given for 20 minute periods starting the third day after birth and lasting for 7 consecutive days. On the day of birth, 3 days after operation and Nile Blue Sulphate treatment, it was noted that the wound had not healed. There were no projections and no scab was present at that time.

On day 4, postoperation, a single projection appeared at the site of the third and fourth digital areas while none was apparent at the fifth digital amputated area. By day 8, the wound had healed. On day 9 the single projection at the third and fourth areas developed a depression that continually deepened until on the 14th day post operation, a separation was achieved forming two projections. It was on the 14th day that a projection was apparent at the fifth digital area.

By the 16th post-operative day the longest projection, #3, appeared to be ¾ the length of the intact digit, #2, while projection #4 was shorter than projection #3, and projection #5 was shorter than projection #4.

An X-ray photograph of the treated manus of this animal is shown in Figure 14. This X-ray was taken when this rat was about 100 days old. At about 2 years of age this animal died. A skeletal preparation was made from which a list of measurements were made relating the length of the phalanges of the new projections with the length of intact phalanges of related digits.

Left Paw Treated		*Right Paw Intact*	
Digit #2 (Intact)		Digit #2	
Proximal phalanx	4.5 mm.	Proximal phalanx	5.0 mm.
Projection #3		Digit #3	
Full Length	5.0 mm.	Proximal phalanx	5.5 mm.
Projection #4		Digit #4	
Full Length	3.5 mm.	Proximal phalanx	4.5 mm.
Projection #5		Digit #5	
Full Length	3.5 mm.	Proximal phalanx	5.0 mm.

The appearance of the third, fourth, and fifth proximal phalanges after skeletal preparation was identical in shape to the reorganized phalanges of the group A rats. The surfaces of the third, fourth, and fifth reorganized phalanges were comparatively rough.

Discussion

One of the problems of stimulating regeneration of tissue, be it amphibian tissue or mammalian tissue, appears to be the removal of inhibiting factors at the amputation site at the right time. It was found in the above experiments that scab material remaining at the amputation site of untreated young rats for 9 days is one factor that limits partial regeneration of rat digits. Trypsin-calcium treatments were used to remove scab material 24 hours after amputation of some digits of young rats. Resulting partial regeneration of these digits followed.

In experiments on animals that normally regenerate limbs (Singer, 11) it can be seen that the scab material is naturally discarded at an earlier time and by a different process than is the case at the amputation site of non-regenerating young rat digits. A thin layer of epithelial cells covers the amputation surface within 24 hours after limb removal. The process of phagocytosis removes the gelatinous scab material, among other things, within the following 5 or 6 days. Untreated digital amputation sites of young rats contrast with the above situation by taking nine days for epitheleum to undercut the scab material.

Other investigators interested in problems of restoring regenerative capacity to other kinds of tissue have also had to grapple with the problem of blood clots at the site of tissue removal.

It is now known that after removal of blood clots by such means as the use of tampons, muscle, and hemostatic substances, regeneration of parts removed from mammalian cerebral hemispheres can be induced to take place. Regeneration will not occur if the wound is not cleaned of blood at the site of removal (Polezhaev, et al, 5). Regeneration of mammalian myocardium can be induced to occur if heart hydrolyzates are injected into the experimental animals. One effect of these injections is to induce the occurrence of scar tissue development at an earlier time than it takes to develop in control animals (Polezhaev, et al, 6). This may be compared to early removal

of blood clots at the wound sites. Pyromen injections into a dog helped this animal to partly regenerate some nerves across a transection of its spinal cord. Pyromen in this case inhibits, to some extent, the formation of scar tissue at the site of transection. Months after termination of treatment loss of behavior associated with the degeneration of certain nerve fibers in the transected area was correlated with the infiltration of scar tissue in the same area (Nesmeianova, et al., 1).

In the light of the above discussion, group C animals treated, though for a shorter period of time, with the same solution used on group A animals, are of especial interest. Group C rats did not develop projections at the site of amputation nor did they reorganize proximal phalanges. It now seems plausible to infer that the results given by control group C as reported above were partly due to the fact that the scab was not removed from the amputation site.

A preliminary experiment (unpublished) on 8 newborn rats in which treatment was different from that applied to group A rats in this experiment, gave no evidence of regeneration.

In the preliminary experiment a razor blade was used to amputate two digits from the right manus of each of five rats, and 4 digits from the right manus of each of 3 rats. Streptokinase-Streptodomase Varidase was used with the trypsin and NaCl with the $CaCl_2$ in the removal of the scab material. The NaCl was used because it has been reported as stimulating the regeneration of tissue of limited regenerational capacity (Rose, 7).

About 12 treatments, similar to those described earlier for group A rats, were needed to remove scab material from the amputation site. After 40 days, no projections had developed.

It was found therefore that scab removal alone did not result in partial regeneration of digits. Either the S-S-V and NaCl may have inhibited tissue regeneration, or the use of a razor blade may have in some way interfered with the organization processes leading to regeneration. Therefore, further experiments are suggested in which chemicals and means of amputation are very carefully regulated.

In utero experiments by Nicholas (2) resulted in a complete absence of digital regeneration of 8 rats operated upon at 7 days, 6 days, 4 days, and one day before term. Embryonic fluid was removed from his experimental animals at the time of amputation of the digits. In

my experiment *in utero,* only a drop or 2 from a possible several hundred drops of embryonic fluid was lost during the amputation of the 3rd, 4th, and 5th digits of a rat embryo. It is not therefore possible to conclude that treatment with Nile Blue Sulphate in Tyrode solution was the cause of the partial digital regeneration that occurred. Embryonic fluid may quite possibly contain material contributing to the stimulation of partial regeneration of rat digits.

The results of the experiment described in this paper indicate that a method has been found to investigate the regenerative potentialities of the digits of a mammal *in vivo.* Included in this method is the application of substances that have been used for some years in tissue culture experiments. Okada (3) demonstrated the regeneration of a cartilage matrix from dissociated chondrocytes, *in vitro.* Some of his solutions consisted of trypsin, Tyrode solution, embryonic fluid, and $CaCl_2$. Thus we now have some evidence to support the possibility that work such as Okada's, performed *in vitro,* is applicable to work done on animals *in vivo.*

Conclusions

It is concluded that the trypsin-calcium treatment and the method of amputation used here led to partial regeneration of cut digits including complete regeneration of the cut phalanx in the mani of treated rats. Segmented digits were not produced by the treatment.

Treatment is not effective unless scab material is completely removed during trypsin-calcium treatment.

Partially regenerating digits begin their development independently of the time needed for wounds to heal completely.

There is no regeneration of proximal phalanges of a rat unless partial digital regeneration is taking place at the same time.

Summary

The fourth and fifth digits of 20 two-day-old rats were amputated with a surgical snare that produces a sharp cut. Trypsin and warmed 0.95% $CaCl_2$ solutions were applied to 12 rats at the sites of amputation one day after operation.

New projections developed at each of the digital amputation sites in 10 out of 12 cases. No projections developed in the case of 8 control rats.

Reorganization of cut proximal phalanges took place in association with the 10 projections that were sectioned. No reorganization of proximal phalanges occurred in the four cut phalanges sectioned from control groups.

It is concluded that the method of amputation and the trypsin-calcium treatments stimulated regeneration of the cut proximal phalanges of young rats. No segmented digital regeneration occurred with these treated animals.

Associated experiments showed that variation of the methods used in treatment and of the surgical tool employed could inhibit regeneration, and that regeneration is possible *in utero*.

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CHEMICAL AND METABOLIC CHANGES IN EMBRYONIC LIVER CELLS FOLLOWING TREAMENT WITH ESTROGENS*

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As has been demonstrated in this laboratory, pharmacodynamic doses of estrogens[1] administered to chicken embryos are without effect in eliciting synthesis of serum X_1-phosphoproteins and serum X_2-lipoglycoproteins (1). Such large doses of estrogens do, however, result in the production of low density serum lipoproteins by the embryonic liver, just as they do in the livers of older chicks and adult chickens (2-3).

Because of an anticipated large shift in intracellular metabolism in order to facilitate the production of these abnormal lipoproteins, certain other changes may be anticipated in the estrogen-treated embryonic liver. In an initial assay for such effects, livers from chicken embryos of various stages of development have been examined with respect to degree of hydration, inhibition of growth and cellular division, derangements of lipid composition and changes in content of glycogen.

In addition to investigations carried out on the above mentioned chemical and metabolic parameters, a very preliminary study has been made of the gross intracellular anatomy, before and following estrogen treatment, with special attention given to wet volumes of nuclei, mitochondria and the microsomal fractions of liver cells.

* This paper is based on work performed under contract No. AT-04-1-GEN-12 between the Atomic Energy Commission and the University of California at Los Angeles.

[1] It was not clearly stated in our earlier publication (*Growth*, 1960, 24, 401) that the amount of estrogen injected (5 mg. per animal) appears in all stages to elicit the maximum response of which the animal is capable. Ten mg. gave the same result as did 5 mg. We wish also to point out that the ratio of wet liver volume to circulatory volume is relatively constant over the stages studied.

Materials and Methods

Embryonated eggs of 9, 10, 15, and 18 days incubation and newly-hatched chicks (21 days of incubation) were injected with 5 mg. of estrogenic substances each (Ayerst Co.) and were sacrificed after 3, 4, or 6 days. Saline-injected eggs and chicks served as controls.

Livers were extirpated whole, weighed immediately on a microbalance and then quickly frozen on dry ice. Water content was determined by first disintegrating the thawed tissue in a piston type disintegrator, then adding acetone to a weighed sample of the wet mash and evaporating thrice under a stream of nitrogen at room temperature, finally drying to constant weight in vacuum (4). Total lipid was extracted from the dried solids by the addition of 10 ml. of a 2:1 chloroform-methanol mixture. The extract was purified by Sperry's modification of the procedure of Folch et al. (4) the total lipid being determined gravimetrically. Assay of the proportions of the various major lipid components was made by chromatographic separations on silicic acid following the procedure developed by Fillerup and Mead (5).

Relative content of glycogen was determined by quickly placing weighed portions of frozen minced liver into 50 milliliters of boiling water for 15 minutes, adding glacial acetic acid dropwise to the hot solution until faintly acid, filtering, adding 3 volumes of 95% ethyl alcohol and measuring the volumes of precipitated glycogen in graduated centrifuge tubes (0.1 ml. graduations).

Differential centrifugation of subcellular components and measurements of the wet volumes of these fractions were carried out by procedures detailed in a previous publication (6).

Results

During the course of normal growth, the livers of chicken embryos and chicks were observed to grow larger with age, the most striking increase taking place between 14 and 20 days of incubation (6). Treatment with estrogen resulted in no deviation from normal liver weights in 13- and 14-day embryos and in newly-hatched chicks; but the livers of estrogenized 18-day embryos weighed approximately 15 per cent less than the livers of their controls; and the livers of estrogenized 3-day chicks weighed 28 per cent more than the livers of untreated chicks (Table I).

TABLE I

TOTAL WEIGHTS, RELATIVE WATER, LIPID AND NON-LIPID SOLIDS OF LIVERS FROM CONTROL AND ESTROGENIZED CHICKEN EMBRYOS AND CHICKS

Number of Animals	Age Estrogen Given	Age Liver Removed	Average Wet Weights of Livers (gms)	Water %	Total Solid %	Lipid % of Total Solid	Non-Lipid Solid % of Total Solid
12	9 days	13 days	.115 ± .014	80.6	19.4	15.0	85.0
11	None	13 days	.117 ± .022	80.3	19.7	15.0	85.0
11	11 days	14 days	.169 ± .022				
11	None	14 days	.196 ± .026				
10	15 days	18 days	.506 ± .089	73.0	27.0		
9	15 days	18 days	.422 ± .058	75.0	25.0	27.0	73.0
10	None	18 days	.594 ± .047	67.8	32.2		
9	None	18 days	.494 ± .049	70.0	30.0	30.0	70.0
8	15 days	21 days	.885 ± .103	68.0	32.0	60.0	40.0
5	18 days	21 days	.879 ± .101	70.0	30.0	55.0	45.0
8	18 days	21 days	.897 ± .094	70.0	30.0	55.0	45.0
6	None	21 days	.938 ± .251	67.0	33.0	77.5	22.5
6	None	21 days	.997 ± .155	64.0	36.0	65.0	35.0
8	None	21 days	.830 ± .084	64.4	35.6	64.0	36.0
8	21 days	24 days	1.346 ± .135	63.3	36.7		
12	21 days	24 days	1.347 ± .168	65.0	35.0	24.0	76.0
8	21 days*	24 days		70.0	30.0	19.0	81.0
8	21 days**	24 days		67.2	32.8	19.6	80.4
12	21 days	24 days		65.0	35.0	26.5	73.5
8	21 days	24 days		63.8	36.2	20.1	80.9
8	None	24 days	1.174 ± .082	53.3	46.7		
12	None	24 days	1.130 ± .141	61.7	38.3	33.5	66.5
12	None	24 days		56.2	43.8	34.7	65.3
8	None	24 days		56.6	43.4	25.1	74.9

* Regenerating portion of liver.

** Non-Regenerating portion of regenerating liver.

Levels of confidence for true differences in liver weights between: estrogenized 18 days embryos and control = P 0.01, estrogenized 3-day-old chicks and controls = P 0.01.

The livers of chicken embryos and chicks were found to be decreasingly hydrated with increasing age to the point of hatching. Estrogen treatment resulted in increases in liver hydration with the notable exception of the youngest embryos studied (13-14 days of incubation) (Table I).

Since the livers of the estrogenized 18-day embryos were both more hydrated and of lesser weight than those of normal 18-day embryos, it is probable that cell division in the liver had been inhibited by estrogen treatment in this age group. The increased hydration of livers of estrogenized newly-hatched chicks and the lack of concomitant increases in total weights of these organs, suggests inhibition of cell division in this age group, also. However, estrogen may have induced some other change to make the tissue as heavy as that of the controls (see lipid changes, Table I). Applying similar criteria, there would seem to have been no impairment of cell division in the younger (13-14 day) groups, nor does there appear to have been any detectable inhibition of this process in 3-day-old chicks (injected with estrogens just at hatching). In fact, estrogen may have somehow stimulated cell division in the livers of 3-day-old chicks since the average increase in total weights of these livers was 28 per cent as contrasted to only 8 per cent increase in unit water.

The ratios of total liver lipids to total liver solids increased to the point of hatching and then fell off sharply in the 3-day-old-chicks (Table I). There was no effect of estrogen on this ratio in 13-14-day embryos but in 18-day embryos, in newly-hatched chicks (injected at 18 days of incubation) and in 3-day-old chicks (injected at the time of hatching) estrogen treatment resulted in decreases in total liver lipid (Table I). These decreases in proportions of total lipid were shown by silicic acid chromatography to be due, almost entirely, to decreases in the sterol ester fraction of the total lipid (Table II). Slight increases in triglyceride contents of the livers seemed to have taken place following estrogen treatment.

Data on the total liver glycogen were obtained only for the newly-hatched chicks and the 3-day-old chicks. In both cases the liver glycogen was decreased following estrogen injection (Table III).

Wet volumes of nuclei, mitochondria and microsomal fractions were not detectably altered in livers of previously estrogenized 18-day-old embryos, newly hatched chicks or 3-day-old chicks (Table IV).

TABLE II

CONSTITUENT LIVER LIPIDS (PERCENTAGES OF TOTAL SOLIDS) OF CONTROL AND ESTROGENIZED EMBRYOS AND CHICKS

Number of Animals	Age & Treatment	Sterol Ester %**	Triglyceride %	Sterol %	Phospholipid %
10	18 day (Estrogen)*	12.2	4.6	.1	10.0
10	18 day (Control)	18.5	1.7	.5	9.3
13	21 day (Estrogen)*	30.0 (27.2-32.6)	7.7 (5.1-10.3)	1.0 (0.7-1.4)	16.4 (16.3-16.5)
14	21 day (Control)	44.5 (40.3-48.1)	5.5 (3.4-7.6)	1.0 (0.7-1.5)	13.7 (12.4-15.0)
12	24 day (Estrogen)*	16.0	1.1	0.1	6.5
12	24 day (Control)	25.9	0.8	0.1	6.7

* Each of these groups of chicks was injected with 5 mg of estrogen (Estrogenic substances, Ayerst Co.) 3 days previously.
** Percent of total liver solids.

TABLE III
RELATIVE LIVER GLYCOGEN OF CONTROL AND ESTROGENIZED CHICKS

Number of Animals	Age Injected	Age Liver Removed	Volumes of Glycogen Precipitated (Arbitrary Units)
8	15 days (5mg Estrogen)	21 days	3.4
6	15 days (Saline)	21 days	6.6
8	18 days (5mg Estrogen)	21 days	2.2
8	18 days (Saline)	21 days	4.0
6	18 days (Saline)	21 days	3.95
12	21 days (5mg Estrogen)	24 days	2.1
12	21 days (Saline)	24 days	3.0
12	21 days (Saline)	24 days	2.6
12	21 days (5mg Estrogen)	24 days	2.6
12	21 days (Saline)	24 days	3.5

DISCUSSION

The fact that no changes were detected in the livers of estrogenized 13-14-day embryos, brings into focus the previous finding that increases in serum low density lipoproteins were smallest in estrogen injected embryos of this age group (1). It, therefore, seems possible that the metabolic changes required for this synthesis were of such low order that the tissue was not significantly stressed in other aspects of its intracellular economy. Alterations taking place in the livers of older embryos and chicks, after injection of estrogens could however, be postulated as being primarily due to adjustments that would permit large quantities of low density lipoprotein to be synthesized.[1] Such accommodation could be expressed in diverse ways; for example, (a) the inhibition of cell division seen in the 18-day embryos might be a reflection of the channeling of total available substrates and sources of energy into other processes so that there was insufficient material available for the events leading to cell division,[2] (b) the decrease of stored sterol ester possibly reflects indirect utilization of this material in the synthesis of estrogen induced protein, (c) the slight increase in liver triglyceride may be due to increased synthesis of this material for low density serum lipoprotein (1).

[1] In older chicks adjustment might also have to be made within the cells for the synthesis of X_1-phosphoprotein and X_2-lipoglycoprotein.

[2] On the other hand, the channeling of energy into synthesis of low density lipoprotein might exert a more selective effect on the processes of cell division such as altering certain specific enzyme systems required for cell multiplication.

TABLE IV

WET VOLUMES OF INTRACELLULAR COMPONENTS OF LIVER CELLS

Number of Animals	Age Injected	Age Liver Removed	Nuclear Wet Volume ml/4ml Liver	Mitochondrial Wet Volume ml/4ml Liver	Microsomal Wet Volume ml/4ml Liver
10	15 days (5mg Estrogen)	18 days	.65	1.8	1.4
10	15 days (Saline)	18 days	.40	1.6	1.6
9	15 days (5mg Estrogen)	18 days	.60	1.85	1.5
9	15 days (Saline)	18 days	.55	1.7	1.5
5	18 days (5mg Estrogen)	21 days	.65	1.4	1.4
6	18 days (Saline)	21 days	.40	1.6	1.4
8	18 days (5mg Estrogen)	21 days	.30	1.3	1.4
8	18 days (Saline)	21 days	.45	1.2	1.4
12	21 days (5mg Estrogen)	24 days	.75	1.0	1.5
12	21 days (Saline)	24 days	1.00	1.0	2.0
12	21 days (5mg Estrogen)	24 days	.46	1.8	1.0
12	21 days (Saline)	24 days	.68	1.9	1.0
12	21 days (5mg Estrogen)	24 days	.68	1.6	
12	21 days (Saline)	24 days	.58	1.2	

Comparison of wet volumes above can only be made in Estrogen—Control pairs due to differing storage and centrifugal treatments.

It is possible that synthesis of low density lipoprotein is not in any way responsible for other changes observed in the liver cells, but that at least some of these changes are, in themselves, directly provoked by injection of estrogens.

Although cell division of the 18-day liver appeared to be inhibited by estrogens, similar treatment of the 3-day-old chick was followed by increased cell division in the liver. This was possibly because the 18-day liver was operating at the upper limit of its cellular reserves (cell division is proceeding very rapidly at this stage) whereas the 3-day-old liver, being more differentiated and containing more substrate was better equipped to meet the estrogen challenge.

A possibly perplexing aspect of this study is that despite the observed changes in hydration of the liver cells in estrogenized animals, there were no consistent changes in the wet volumes of nuclei, mitochondria or microsomal fractions. Apparently the analyses were not sufficiently precise to distinguish changes that averaged only 7 per cent for the entire organ. Supplementary studies with the electron microscope are now under way.

Summary

These studies show additional effects of estrogen treatment on the physiology of the liver of the chicken embryo and chick.

With the exception of the youngest embryos studied (13-14-days of incubation) the livers of all estrogen treated animals contained higher percentages of water than their respective controls.

Rates of cell division in the livers of 18-day-old chicken embryos appeared to be inhibited by estrogens but such inhibition did not seem to occur in the livers of 13-14-day-old embryos or 3-day-old chicks. In fact, the rate of cell division was probably increased in the 3-day-old chicks.

Ratios of total liver lipids to total liver solids were decreased by estrogen treatment in all stages studied except the youngest (13-14-days of incubation). These decreases were due to decreases in sterol esters. Liver glycogen was decreased following estrogen injection of older stages, but wet volumes of nuclei, mitochondria and microsomal portions were not detectably altered.

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EFFECTS OF ESTROGENS ON PROTEIN-BOUND CARBOHYDRATES OF EMBRYO AND CHICK SERUM*

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Since serum X_2-lipoglycoprotein (lipovitellin) is increased only slightly in chicks injected with estrogenic substances (1-3) and not at all in embryos thusly treated, it was expected that very little change would take place in the total serum glycoprotein[1] of these animals. However, preliminary analyses for protein-bound carbohydrates of serum in estrogen injected chicks have revealed large elevations in amounts of total serum glycoprotein whereas the seromucoid[2] fraction is considerably depressed.

From the above results several questions have arisen regarding the specific nature of the changes. (a) Is the additional protein-bound carbohydrate associated with any proteins of the serum other than X_2-lipoglycoprotein? (b) If so, can the increase in total serum glycoprotein and the decrease in seromucoid be induced in the embryo as well as in the chick? (c) Are the increases in total serum glycoproteins proportional to the decreases in seromucoids under various circumstances, including different stages of development and varying lapses of time following injections of estrogens? These questions are treated in the present paper.

MATERIALS AND METHODS

Embryonated eggs of 15, and 18 days of incubation and newly hatched chicks received 5-6 milligrams Estrogenic Substances (Ayerst

* This paper is based on work performed under contract No. AT-04-1-GEN-12 between the Atomic Energy Commission and the University of California at Los Angeles.

[1] The non-glucosamine polysaccharide component or hexose moiety of the serum glycoproteins precipitated by an ethanol concentration of 90 per cent by volume at 25°.

[2] The non-glucosamine polysaccharide component or hexose moiety of the perchloric acid-soluble, phosphotungstic acid-insoluble proteins occurring in serum. Seromucoid hexose comprises part of the total serum glycoprotein.

Co.) each to the yolk sac. Certain controls for each age group were injected with saline. Embryos injected at 15 days were bled on the 18th or 21st day; embryos injected at 18 days were bled 3 days later (on the day of hatching) and chicks injected after hatching were bled 2, 4, 6 and 7 days following initial treatment. One portion of the serum in each case was diluted 1:1 with 17.6 per cent NaCl so that the total ultracentrifuge pattern could be ascertained in the analytical ultracentrifuge (see Fig. 1). The remainder of the serum was frozen and stored for analysis of total serum glycoprotein (4) and seromucoid (5) (see Fig. 1, Tables I and II). Since the proportions of the various components of the ultracentrifuge pattern changed considerably as a function of the stage of development and the time elapsed following initiation of estrogen treatment, comparisons of their concentrations with the rise and fall of total serum glycoproteins and seromucoids could be made.

In one experiment, the total serum proteins of a group of 23-day-old estrogenized chicks were divided into two vertical fractions by ultracentrifugation in a NaCl medium of density 1.063 (70,000 g for 24 hours) so that all of the light lipoprotein elicited by estrogen treatment was concentrated in the top fraction (1/3 of the total volume). Analysis of total serum polysaccharide and seromucoid was made on this fraction in the same manner as for the whole serums (Table III).

Results

The largest increases of total serum glycoproteins were observed in estrogenized young chicks 2-7 days post-hatching[3] (Figure 1, Table I). The largest decreases in seromucoid also took place in estrogenized young chicks (Table II). Eighteen-day-old embryos and newly hatched chicks displayed smaller elevations in the protein-bound carbohydrate after estrogen treatment and likewise showed lesser decreases of the seromucoid fraction (Tables I and II).

Elevation of polysaccharide in the cases of 18-day and newly-hatched chicks could not be attributed to the presence of X_2-lipoglyco-

[3] Injected with 5 mg Estrogenic Substances on the day of hatching. Previous experience has shown no sex differences in chicks in response to estrogens. Also, 5 mg. of Estrogenic Substances per animal results in a maximal response in embryos and chicks of these ages. Ratios of liver wet volume to total circulatory volume are approximately equal within the age groups in this study.

TABLE I

RATIOS OF INCREASE IN SERUM LIGHT LIPOPROTEIN (S_f10-100) TO INCREASE IN TOTAL SERUM POLYSACCHARIDE

Age Est.* Given	Bled Age	S_f 10-100 (Arb. Units)**	Total Observed Hexose (mg %)	— Average Total Hexose of Controls (mg %)	+ Decrease in Seromucoid Hexose (mg %)	= Increment of Increase (mg %)	Ratio S_f 10-100 (Arb. Units) : Increased Hexose (mg %)
15 d	18 d	16	29.2	25.0	0.0	4.2	1 : .26
15 d	18 d	20	33.0	25.0	0.0	8.0	1 : .40
15 d	21 d	48	60.0	50.0	5.1	15.1	1 : .31
18 d	21 d	80	70.6	50.0	2.7	23.3	1 : .29
21 d	23 d	124	90.8	65.0	5.0	30.8	1 : .25
21 d	23 d	140	96.8	65.0	3.7	36.5	1 : .26
21 d	23 d	160	111.2	65.0	4.8	51.0	1 : .32
21 d	25 d	168	105.1	65.0	3.7	43.8	1 : .26
21 d	25 d	148	88.0	65.0	6.8	29.8	1 : .20
21 d	27 d	104	88.0	62.0	6.7	32.7	1 : .31
21 d	27 d	96	90.6	62.0	2.7	31.3	1 : .32
21 d	28 d	84	87.4	62.0	3.3	28.7	1 : .34
21 d	28 d	124	90.4	62.0	5.5	33.9	1 : .27

	Total Hexose	Seromucoid Hexose	
Controls 18 d	25.0 ± 2.3	4.0 ± 0.8	Geometric Mean 1 : 0.29
Controls 21 d	50.0 ± 3.3	14.0 ± 1.3	
Controls 24 d	65.0	14.0	
Controls 28 d	62.0	14.0	

* Each analysis of estrogenized embryos and chicks was performed in duplicate on a minimum group of 10 animals. Control values were averages of current and previous analyses (6) (7).

** Arbitrary Units are equal to 1 square millimeters of deflection caused by light lipoprotein in ultracentrifuge pattern of undiluted serum (8.8 per cent NaCl added).

FIGURE 1. Representative ultracentrifuge patterns of whole embryo and chick serums obtained at various intervals after treatment with estrogens. The frames to the left were taken 10 minutes after reaching speed (190,000 g), those in the middle were taken 42 minutes after reaching speed, and those on the right were taken 106 minutes after reaching speed. The patterns, featuring the light lipoproteins (l.), on the left are upside down due to reversal of the bar angle. Deflection of light lipoproteins is negative at this NaCl concentration (8.8 per cent) and the bar angle was reversed to better keep the peak within the limits of the frame. Areas of deflection from the horizontal line, best seen in the patterns to the left, are generally proportional to the concentration of those components. (a) refers to albumin, (X_1) to X_1-phosphoprotein, (X_2) to X_2-glycolipoprotein, and (d.l.) to dense lipoprotein (-s 0-10). Protein-bound serum hexose values represent increases over control values (in mg %) and seromucoid hexose values represent decreases over control values. Other experiments of this type gave similar results.

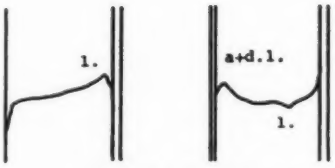
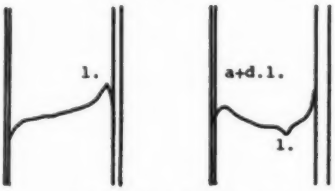
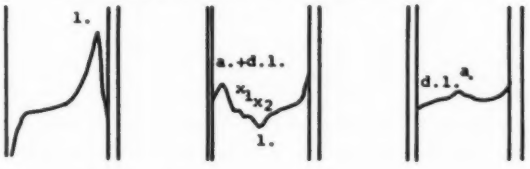
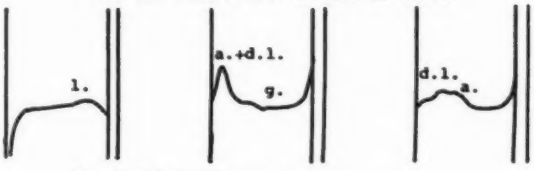
	POLYSACCHARIDE	
	Total Hexose (gain)	Seromucoid Hexose (decrease)
 18-DAY-OLD EMBRYO, EST. ON 15th DAY	8.0	0
 21-DAY-OLD EMBRYO, EST. ON 15th DAY	15.1	5.1
6-DAY-OLD CHICK, EST. ON DAY OF HATCH	26.0	6.7
 7-DAY-OLD CHICK, EST. ON DAY OF HATCH	28.4	5.5
 7-DAY-OLD CHICK, NO ESTROGEN	0	0

TABLE II
RATIO OF PERCENTAGE INCREASE OF TOTAL SERUM POLYSACCHARIDE TO PERCENTAGE DECREASE IN SEROMUCOID FRACTION

Age Est. Given	Age Bled	Per Cent Increase Total Serum Hexose	Per Cent Decrease Serum Seromucoid	% Total Hexose : % Seromucoid Hexose
15 d	18 d	32.0	0	1 : 0.00
15 d	18 d	16.8	0	1 : 0.00
15 d	21 d	30.2	36.5	1 : 1.20
18 d	21 d	46.7	19.3	1 : 0.41
21 d	23 d	47.6	35.7	1 : 0.75
21 d	23 d	56.2	26.7	1 : 0.47
21 d	23 d	78.8	34.2	1 : 0.43
21 d	25 d	67.4	26.4	1 : 0.39
21 d	25 d	44.4	48.5	1 : 1.09
21 d	27 d	52.8	47.8	1 : 0.90
21 d	27 d	50.5	19.3	1 : 0.38
21 d	28 d	46.4	23.5	1 : 0.50
21 d	28 d	54.7	39.3	1 : 0.71
		Mean = 48.0	Mean = 27.5	Mean = 1 : 0.56

TABLE III
RESULTS OF ANALYSIS OF ISOLATED LIGHT LIPOPROTEIN (S_f10-100)

Material	Total Serum Polysaccharide (mg % of Hexose)	Total Seromucoid (mg % of Hexose)	Total Light Lipoprotein (mg/ml)
Whole Serum, 2-day-old chick (Estrogenized)	111.1-111.3	8.8-9.6	
Top 1/3 of Ultracentrifuge Tube*	196.0-228.0	6.7-7.5	264-268
Normal Serums, 2-day-old chick	65**	14.0**	9**

* Centrifuged Whole Serum from 2-day-old chick (Estrogenized).
** Averaged from Previous Experiments (6) (7) (8).

protein in the serum because this material was absent in these animals, appearing first in chicks sacrificed at 2 days or more post-hatching. It was noted, however, that in *every instance regardless of age,* where an increase was seen in total serum glycoprotein, a corresponding increase of low density lipoprotein (S_f10-100) was present (see ultracentrifuge patterns, Figure 1).

The ratios of increase of light lipoprotein (Arb. units) to increase in total serum glycoprotein (mg %) for estrogenized embryos and chicks are shown in Table I. This ratio was sufficiently consistent (percentage standard error for the ratios = 3) so that a definite

correlation was established ($P<.05$) between elevation of light lipoprotein and elevation of serum glycoprotein.

Further corroboration of this relationship was obtained when it was discovered that the light lipoprotein isolated by ultracentrifugation from the serums of 2-day-old chicks contained all of the additional protein-bound hexose appearing in the serum subsequent to injection of estrogen (Table III).

Per cent decreases of seromucoid in the different estrogenized groups are presented in Table II. These decreases averaged less in per cent (Mean decrease = 27.5%) than the per cent increases in total serum glycoprotein (Mean increase = 48.0%). Ratios of seromucoid decreases to per cent increases in total serum glycoprotein showed extreme variability from group to group (Table II).

Discussion

The consistent ratios of increased serum light lipoproteins (S_f10-100) to increased total serum glycoproteins (Table I), plus the results of analysis of the light lipoprotein isolated by ultracentrifugation (Table III) indicate that the extra protein-bound polysaccharide in the serum following estrogen treatment is largely bound to lipoproteins of this diffuse group.

In the calculations of increments of increase in total serum glycoproteins, allowance was made for the decreases in the seromucoid fraction (Table I). However, no corrections were applied for the increase in total serum glycoprotein due to X_2-lipoglycoproteins or for decreases due to decreased dense lipoprotein (-S 0-1O). The two factors were ignored because they cancel out. Both changes are lesser or greater in the same animals under the same circumstances and both changes are relatively small in terms of total polysaccharide involved.

Although it is not probable that the distribution of polysaccharide is uniform throughout the density range of light lipoproteins, if the *ratio of polysaccharide to polypeptide* may be assumed to be fairly constant, calculations of the amount of polysaccharide associated with a given weight of the protein component of the light lipoproteins can be made. First, the extra lipid associated with estrogenized serum can be ascertained and compared to the extra deflection of light

lipoprotein in the ultracentrifuge pattern.[4] (This lipid is assumed to comprise 83 per cent of the total lipoprotein of the S_f10-100 density class (8)). In ultracentrifuge patterns of undiluted serum, 1 square millimeter of the deflection due to light lipoprotein is equivalent to approximately .75 mg/ml of whole light lipoprotein or 13 mg/ml of the protein moiety alone. Thus, it can be calculated from the ratios in Table I that, on the average, 1 mg of the protein moiety of light lipoprotein (S_f10-100) contains approximately .038 mg of hexose.

Second, reference can be made to the analysis of light lipoprotein hexose depicted in Table III. Here:

(a) Total lipid per ml of top fraction = 227.5 mg.[5]

(b) total lipoprotein polypeptide per ml of top fraction = 17 per cent of 227.5 mg = 39.0 mg.

(c) Total polysaccharide per ml top fraction = 2.16 mg.

(d) Non-light lipoprotein bound polysaccharide = .10 mg/ml (Estimate).

Thus: approximate mg hexose per mg polypeptide moiety of light

$$\text{lipoprotein} = \frac{c—d}{b} = \frac{2.16—.10}{39.0 \text{ mg}} = .053 \text{ mg.}$$

Just as it is obvious that increased total protein-bound hexose is largely associated with the increased light lipoprotein in estrogenized embryos and chicks, so it is evident that seromucoid hexose is neither associated directly nor closely related to this lipoprotein fraction. Although seromucoid protein decreased as the total polysaccharide increased, the ratio of decrease of seromucoid to increase in total serum polysaccharide varied greatly from group to group. The decrease in seromucoid was, in fact, not closely correlated with any of

[4] This can only be done in the case of chicks since embryo serum contains large amounts of lipid associated with proteins that are lighter than the S_f10-100 density class.

[5] Some of the total lipid measured in this case, as well as in the case of lipid of whole serums of estrogenized chicks, does not fall into the S_f10-100 density class. However, the error caused by this occurrence cannot be great since (in the chick) relatively small amounts of lipid are associated with lipoproteins on either side of this range and they thus tend to cancel out.

the proteins resolvable by ordinary techniques in the ultracentrifuge. The decrease in seromucoid would (in both embryos and chicks) seem to be a non-specific adjustment of the cell economy in favor of producing estrogen stimulated proteins such as light lipoproteins, X_1-phosphoprotein and X_2-lipoglycoprotein.

Increases in total serum glycoprotein and decreases in seromucoid fraction occurred in the embryo as well as in the chick but the increases of both S_f10-100 lipoproteins and total serum polysaccharide were smaller. In the 18-day-old embryos and in newly hatched chicks that had been estrogenized on the 15th day of incubation, there was considerable additional lipid present in a low density class that contained very little polypeptide and polysaccharide. Thus, the livers of estrogenized younger animals reacted to estrogen principally with a lipid response and appeared to have a lesser propensity for producing protein-bound carbohydrate.

The fact that polysaccharide was in every case associated with the light lipoprotein (S_f10-100) and in relatively definite ratio suggests that polysaccharide may be an integral part of this molecule.[6]

Summary

Following injection with Estrogenic Substances young chicks (2-7 days post-hatching) displayed large increases in total serum glycoprotein. Embryos reacted less strongly in terms of total protein-bound carbohydrate but showed a significant lipid response. The increase of polysaccharide was always associated with an increase in light lipoprotein (S_f10-100). In fact, since the ratio of protein-bound carbohydrate to light lipoprotein seemed so constant, even in prehatching stages, it seems possible that polysaccharides constitute an integral and perhaps necessary component of the total light lipoprotein moiety, whether the latter is estrogen induced or not.

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[6] Since the above study, additional analyses of centrifugally isolated light lipoproteins from both control and estrogenized chicks have been carried out in Dr. Henry Weimer's Laboratory, U.C.L.A. These revealed the presence of hexose in similar proportion in both types of lipoprotein (approx. 0.04 mg/mg protein) but the estrogen-induced light lipoprotein appeared to contain 25 to 50 per cent less hexoseamine (0.01 mg), sialic acid (0.01 mg) and fucose (0.005 mg) per unit weight (1.0 mg) of polypeptide. Periodic-Schiff reagent (specific stain for carbohydrate) stains both of these lipoproteins.

There occurred a less consistent decrease in seromucoid fraction in all stages estrogenized and this appeared to be unrelated to any of the serum proteins resolved by ultracentrifugation.

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THE EFFECT OF TEMPERATURE UPON THE OVARIAN TUMORS OF THE *FES* MUTANT OF *DROSOPHILA MELANOGASTER*[1]

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INTRODUCTION

The developing egg of *Drosophila melanogaster* consists of a 16 cell nest surrounded by an envelope of follicle cells. The 16 cells are daughters which arise from four consecutive divisions of an oogonium. Fifteen of the daughter germ cells differentiate into nurse cells and nourish the most posterior, daughter germ cell which becomes the oocyte. The chromosomes of the nurse cells elongate by uncoiling and concurrently undergo a series of 8 or 9 endomitotic doublings which produce polytene chromosomes whose multiple strands become more and more loosely associated as the nucleus grows. Intercommunication of cytoplasm between all members of the 16 cell cyst is made possible by large pores in the walls separating adjacent cells. At first all 16 germ cells grow at roughly identical rates. However, once vitellogenesis begins the oocyte grows at a rate 10 times faster than previously and at the expense of the nurse cells which shrink and eventually degenerate (King, Rubinson, and Smith, 1956; King, 1957a; King and Devine, 1958; King, 1960).

Hereditary ovarian tumors occur in female *Drosophila* homozygous for the recessive genes *fused* or *narrow*[2] or *female-sterile* (King, Burnett and Staley, 1957). In the case of the second chromosomal gene *female-sterile (fes)*, the ovarioles of homozygous females are subdivided into a series of sausage-shaped cell aggregates, each surrounded by an ill-defined follicular epithelium. The follicles are generally filled with hundred to thousands of oogonia-like cells, and

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many of these cells are in various stages of mitosis. Some metaphases are multipolar and others show high degree of polyploidy. It has been shown (King, Sang, and Leth, 1961) that some cells within tumorous chambers in ovaries of certain ages (especially during the second and third week of adult life) can differentiate into cells which, on the basis of chromosomal morphology, resemble normal nurse cells. A positive correlation exists between the frequency of this more normal differentiation and the degree to which the residual genotype is heterozygous. Banded, polytene chromosomes are observed in the nuclei of some of the larger, nurse-like cells. Finally the above workers demonstrated that the *fes* gene when homozygous does not confer special growth factor requirements upon fly larvae. In the studies mentioned above the flies were reared at 25° C. The present communication will give consideration to the effect of a variety of temperatures upon the growth and differentiation of the ovaries of *fes* females.

Methods

Females of genotype S fes + + Alu lt/ + fes dp ^{tx} Sp + + arising from the cross S fes Alu lt/ al^2 Cy (In 2 L R) cn^2 L^4 sp^2 female X fes dp ^{tx} Sp/ S^2 Cy (In 2 L R) cn^2 L^4 sp^2 male (or the reciprocal cross) were used as experimental material. The flies were reared upon *Drosophila* medium (see King and Wood, 1955 for recipe) and were kept in refrigerated incubators at 18, 21 or 25° C. Flies were grown at one of the three temperatures for the larval, pupal and adult period. Embryogenesis occurred at 25° C. in all cases. At 25° C. preadult development takes 9 days. At 21 and 18° C. this developmental period is increased by 6 and 15 days, respectively. Feulgen-stained preparations of ovaries from flies of various ages were made according to the procedure of King, Burnett and Staley, 1957, pg. 242. A cytological study was made of a total of approximately 16,000 chambers.

Results and Discussion

The production of nurse-like cells

Three types of chambers are seen in the ovaries of *fes/fes* females: chambers containing *only* undifferentiated tumor cells, chambers with tumor cells *and* nurse-like cells and chambers containing *only* nurse-

like cells. On rare occasions chambers of the latter type will also contain an oocyte. The frequency of this occurrence is about 2% in non-tumorous chambers from the 18° C. series. The relative proportions of the three types of chambers were found to vary as a function of temperature (see Figure 1). A total of 913, 3771 and 1854 chambers were observed in the 25, 21 and 18°C. series, respectively. There is an inverse relationship between temperature and more normal differentiation, since the frequency of chambers containing only nurse-like cells rises markedly with decreasing temperature, while the frequency of chambers containing only tumor cells rises with increasing temperature.

Next a study was made of the distribution of chambers containing a given range of nurse cell numbers within populations of tumorous or non-tumorous chambers of varying sizes from the ovaries of *fes/fes* flies reared at 18° C. (see Figure 2). In young flies (age 0-5 days) roughly half the chambers had 11-15 nurse cells, and relatively few chambers had smaller or larger numbers of nurse cells. In older ovaries (from flies 15-21 days old) chambers were found to show a greater spread of "nurse" cell numbers. The model class was 16 or more. In old ovaries some fusions of adjacent chambers appear to occur. Such fusions are responsible in part for the excessive numbers of nurse cells observed. The distribution of chambers containing various numbers of nurse-like cells was similar for a given age group whether tumorous or non-tumorous chambers were considered. Thus, the presence of tumor cells has little effect upon the differentiation of some cells into "nurses".

A comparison of the kind just described was made of flies from the 21° C. series. Flies aged 15, 21, 22, 23, 33 and 34 days showed similar distributions, so the data were lumped (see Figure 3). By contrasting Figure 3 and Figure 2 (upper) it can be seen that the main difference between the 18° and 21° C. series resides at the right portion of the curves. In the 18° C. series a larger proportion of the chamber population has 16 or more "nurse" cells per chamber than does the 21° C. series. In view of the above finding a comparison seemed worthwhile between flies from the 18, 21 and 25° C. series using the criterion of the frequency of chambers with 16 or more "nurse" cells. This was done for flies 15-21 days in age (see Table 1).

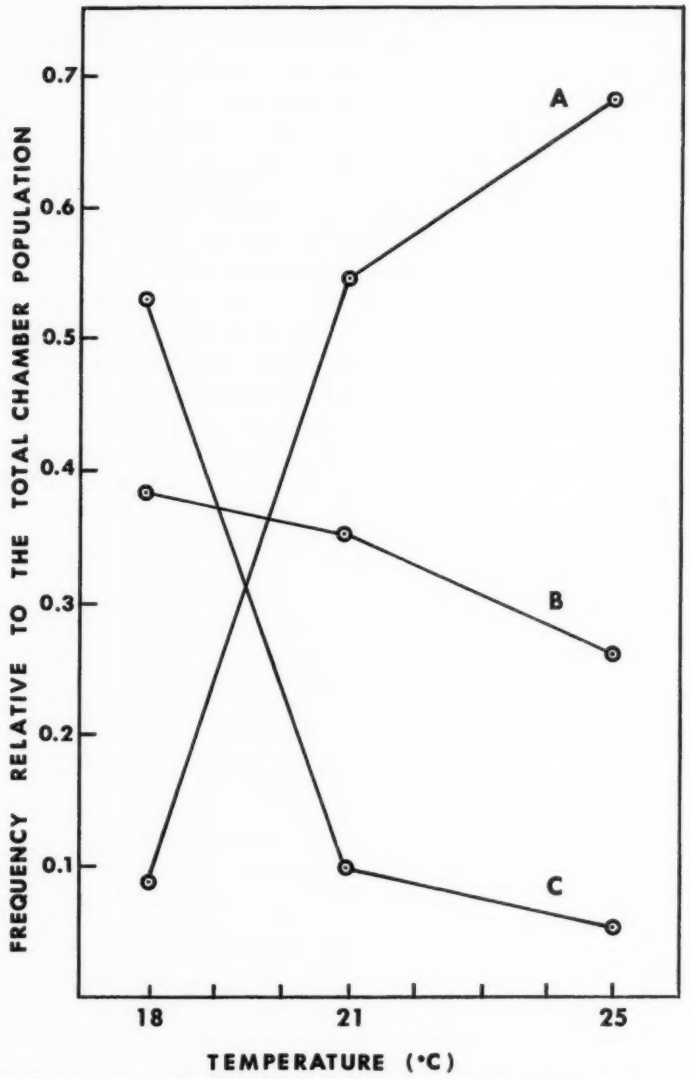


FIGURE 1. The relative proportions of different types of chambers in the ovaries from *fes/fes* flies reared at a variety of temperatures. Curves A, B, and C show the relationships for chambers containing *only* tumor cells, chambers with tumor cells *and* nurse-like cells, and chambers containing *only* nurse-like cells, respectively.

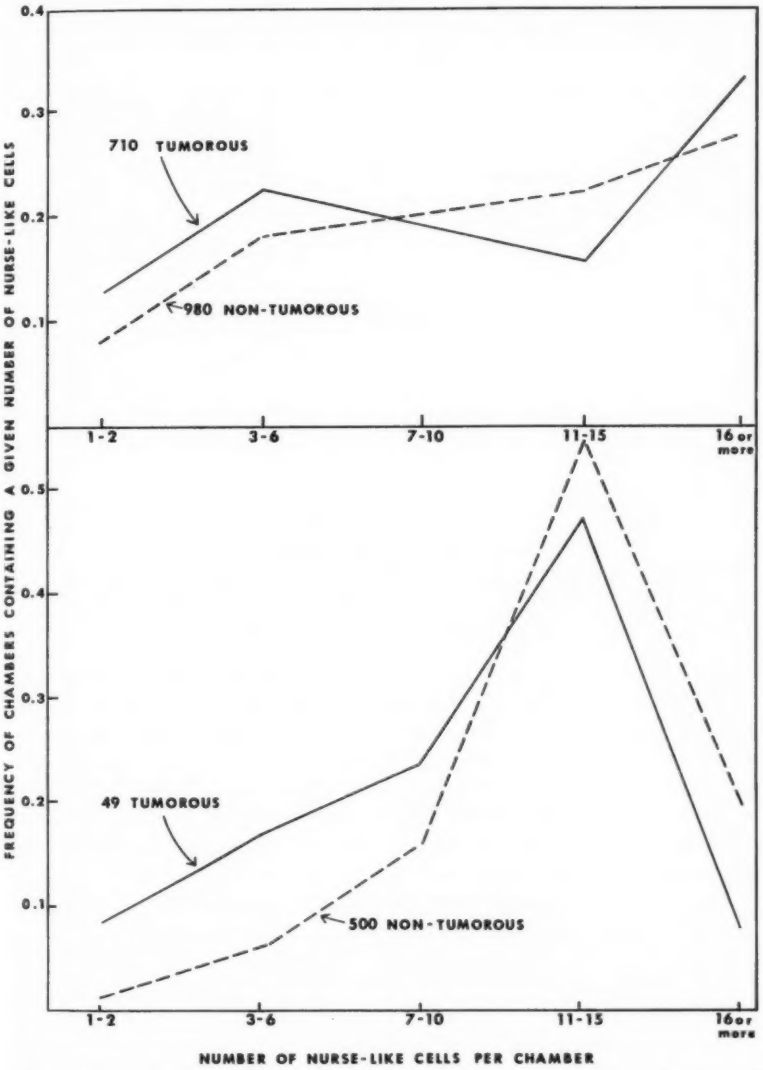


FIGURE 2. The distribution of chambers containing a given range of nurse cell numbers within populations of tumorous or non-tumorous chambers of varying sizes from the ovaries of *fes/fes* flies reared at 18° C. The numbers adjacent to the arrows give the number of chambers observed in each population. The upper curves are for chamber populations from flies 15-21 days old; the lower curves for flies 0-5 days old.

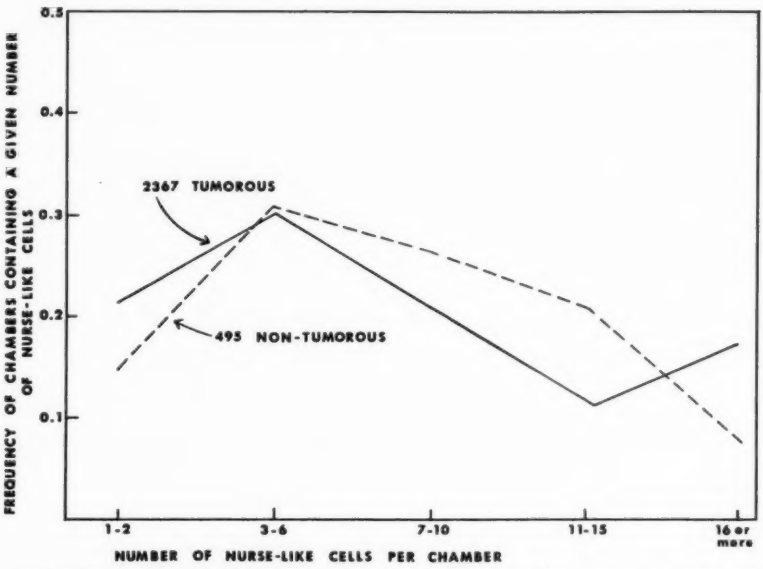


FIGURE 3. The distribution of chambers containing a given range of nurse cell numbers within populations of tumorous or non-tumorous chambers of varying sizes from the ovaries of *fes/fes* flies 15-34 days old reared at 21° C. The numbers adjacent to the arrows give the number of chambers observed in each population.

Again there is an inverse relationship between temperature and "nurse" cell production, and similar frequencies are found for tumorous and non-tumorous chambers. Thus at lower temperatures not only do more chambers contain "nurse" cells, but more "nurse" cells occur per chamber. Furthermore, the effects with respect to temperature are not strictly linear, since the values for the 21° C. series are much closer to the 25° than to the 18° values (see Figure 1 and Table 1).

Next the relationship was determined between the number of "nurse" cells per chamber and the volume of the "nurse" cell-containing chambers (see Figure 4). The volumes were calculated by substituting the appropriate values into the equations $v = 0.5236\ d^{3}$ and $v = 0.5236\ w^{2}\ l$, where $w =$ the width and $l =$ the length of elliptical chambers and $d =$ the diameter of spherical chambers. No attempt was made to correct for shrinkage of tissue during processing.

TABLE I

FREQUENCIES OF CHAMBERS WITH 16 OR MORE NURSE CELLS IN *Fes/Fes* FLIES 15-21 DAYS IN AGE REARED DURING THE LARVAL, PUPAL AND ADULT STAGES AT VARIOUS TEMPERATURES

Temperature (° C)	Non-tumorous chambers		Tumorous chambers	
	$\bar{x}$	N	$\bar{x}$	N
18	0.272	980	0.327	710
21	0.091	375	0.136	1330
25	0.061	33	0.135	245

$\bar{x}$ = average frequency.
N = size of chamber population.

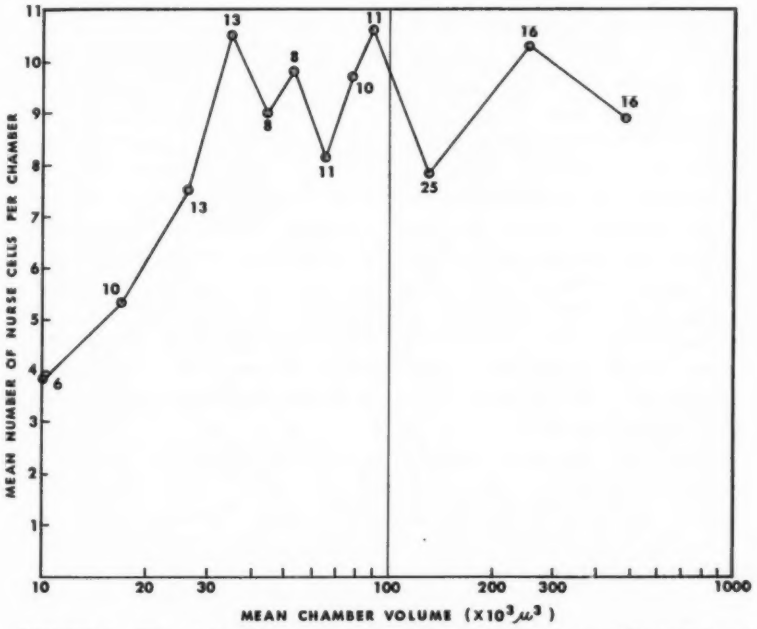


FIGURE 4. The relation between chamber volume (expressed logarithmically) and the number of nurse cells per chamber. The number beside each point gives the sample size. Flies 15 days old, reared at 18 or 21° C. Both tumorous and non-tumorous chambers are included in each sample.

The smallest chambers observed were similar in size to stage 2 egg chambers (see King, Rubinson and Smith, 1956, for the descriptions of the stages in normal oogenesis of *Drosophila melanogaster*). The average number of "nurse" cells per chamber increases by a two-fold factor as the chamber volume triples. Once a volume of 3×10^4 μ^3 (equivalent to a stage 4 egg chamber) is exceeded, the average number of "nurse" cells remains relatively constant. However, the "nurse" cell nucleoplasm and cytoplasm do increase in volume. These findings suggest that the differentiation of "nurse" cells occurs at an early stage during the growth of the chambers.

Chambers containing nurse-like cells fall into two classes: (1) those containing an assemblage of "nurse" cells whose nuclei are homogeneous in size (see Figure 5) and (2) those which contain "nurse" cells whose nuclei vary greatly in size (see Figure 6). The former class is about four times more abundant. In class 2 chambers large and small nuclei sometimes seem to be random in their distribution within the chamber. In other chambers "nurse" cells with nuclei of roughly the same size are clustered together. As was mentioned earlier, in older ovaries adjacent chambers sometimes fuse, and such fusions are responsible in part for chambers with a heterogeneous assemblage of nurse cells. However, class 2 chambers are found in young ovaries, and both class 1 and class 2 chambers exhibit a similar spread of volumes. The fact that one finds tiny class 2 chambers in young ovaries shows that different nurse-like cells in a single ovarian chamber of a *fes* fly often for no apparent reason grow at rates which differ considerably.

Class one chambers contain varying numbers of "nurse" cells. Generally chambers with relatively few "nurse" cells display larger "nurse" nuclei than do chambers with large numbers of nurse-like cells. For a given temperature the largest "nurse" nuclei are generally found in chambers from the oldest flies (34 days was the maximum age observed). It is in these large nuclei that banded, polytene chromosomes are found. However, the chambers from older flies have fewer "nurse" cells per chamber, and degenerating "nurse" cells are plentiful in old chambers. Non-staining spherical vesicles are commonly found imbedded in tumorous chambers of 4-week old flies. Such vesicles mark the position of "nurse" cells which have degenerated completely.

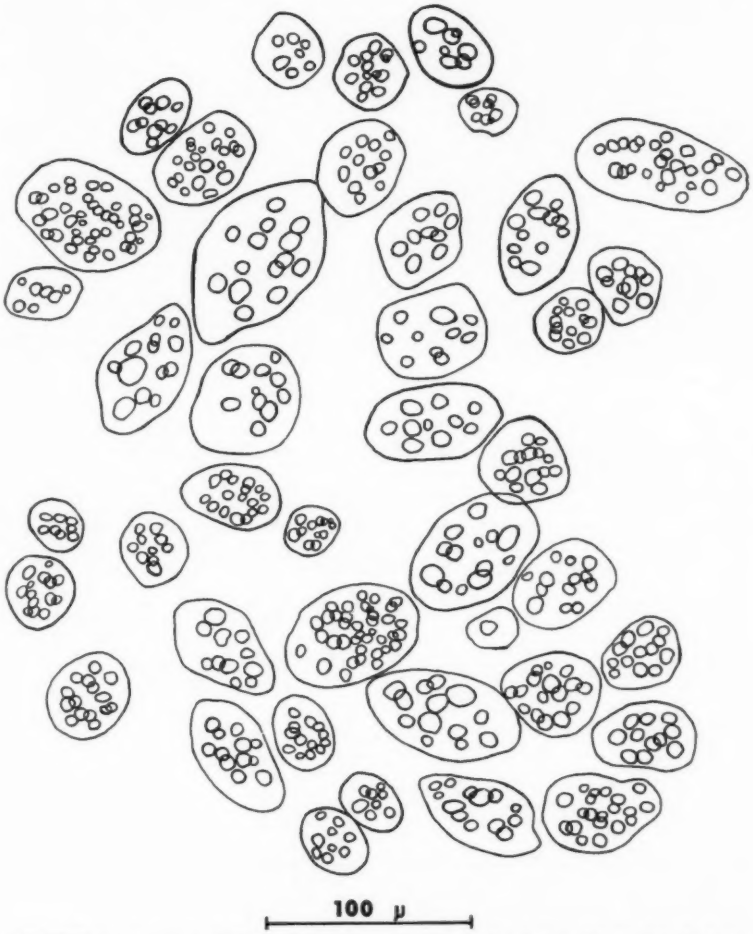
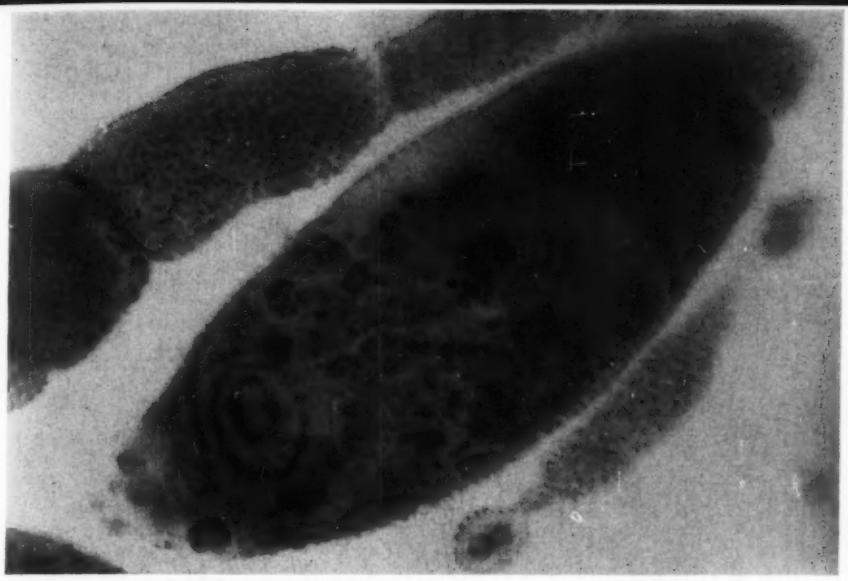
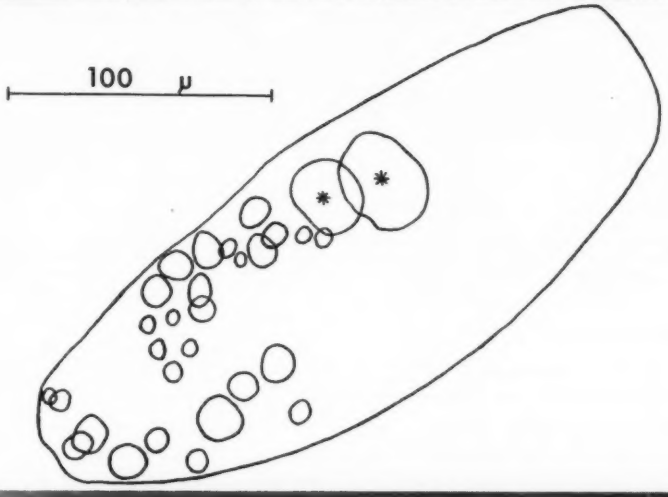


FIGURE 5. A group of 41 chambers from the ovary of a 1 day old, *fes* female from the 18° C series. In this outline drawing only the chamber border and the nuclear envelopes of the nurse-like cells are drawn. The follicle cells are omitted. The average number of nurse-like nuclei per chamber is 13.2. The range is between 1 and 39. No oocytes were present in any of the chambers.

FIGURE 6. The largest tumorous chamber observed. The upper and lower portions of the figure consist of photomicrographs taken at two focal planes. The middle drawing outlines the nuclear envelopes of the nurse-like cells. The nuclei of the tumor cells are not shown. Thirty nurse nuclei are present, and the two largest contain banded, polytene chromosomes. These nuclei are marked with asterisks in the drawing. The tumor which was found in a 33 day old, *fes* female from the 21° C series is approximately the size of the late stage 10 egg chamber seen in Figure 9.

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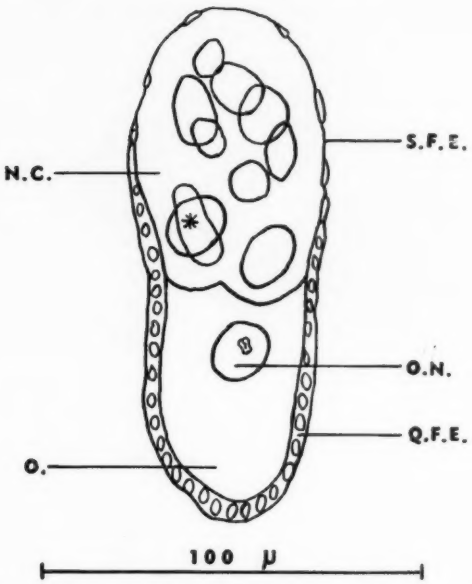


FIGURE 7. An early, stage 10 chamber. Ten nurse cells are present, and one contained banded, polytene chromosomes. The nurse chamber (N.C.) is surrounded by an epithelium of squamous follicle cells (S.F.E.). The ooplasm (O.) which is surrounded by an epithelium of cuboidal follicle cells (Q.F.E.) contains a nucleus (O.N.).

Vitellogenesis

As was mentioned earlier, in the majority of chambers containing nurse-like cells no oocytes are present. However, oocytes are observed rarely, and when present yolk is often laid down in them. The frequency of yolky oocytes among chambers from flies reared at 18° C. is 80 times that found in the 25° C. series (see Table 2). Oocytes in stages 10 and 14 are the most abundant. Karyosome-containing nuclei or karoyspheres have been observed in all the yolky oocytes studied.

TABLE II

Temperature (° C)	F	N	Stages Represented							
			7	8	9	10	11	12	13	14
18	1.59	3,529	1	3	4	17	1	4	5	21
21	0.39	4,132				2		1	1	12
25	0.02	5,440				1				

The frequency (F) of yolky oocytes (expressed as a percent) among populations of chambers from the ovaries of flies reared at a variety of temperatures. N is the size of the sample of chambers.

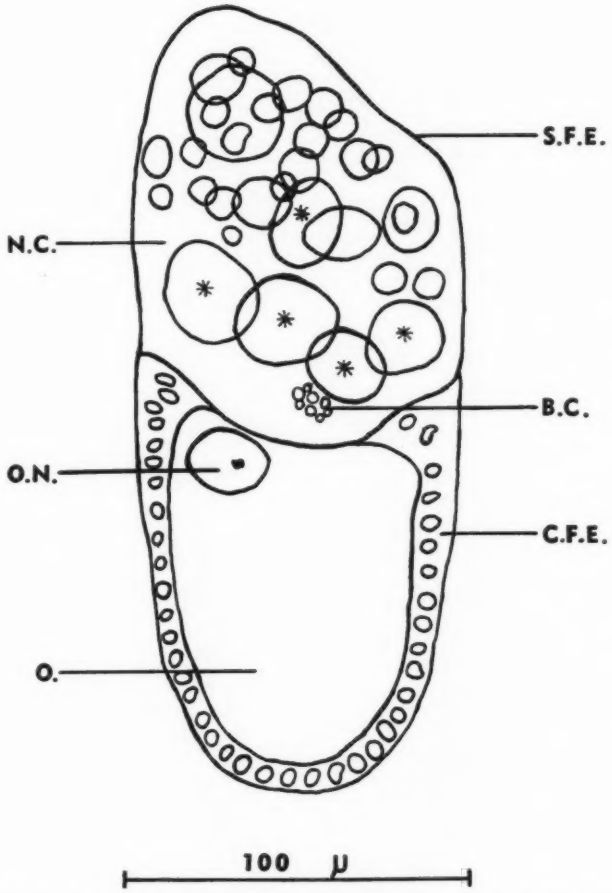


FIGURE 8. A middle, stage 10 chamber. Thirty-one nurse cells are present, and five contained banded, polytene chromosomes. The oocyte is surrounded by an epithelium of columnar follicle cells (C.F.E.). A cluster of border cells (B.C.) is present.

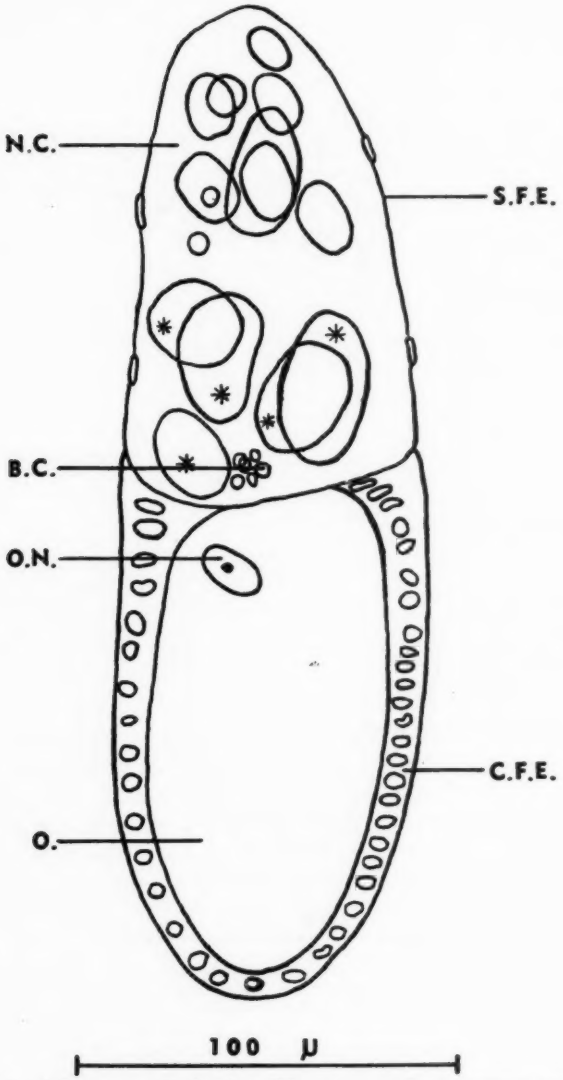


FIGURE 9. A late, stage 10 chamber. Fifteen nurse cells are present, and five contained banded, polytene chromosomes.

FIGURE 10. A photomicrograph of a Feulgen-stained late, stage 10 chamber. This chamber is the one drawn in Figure 9.

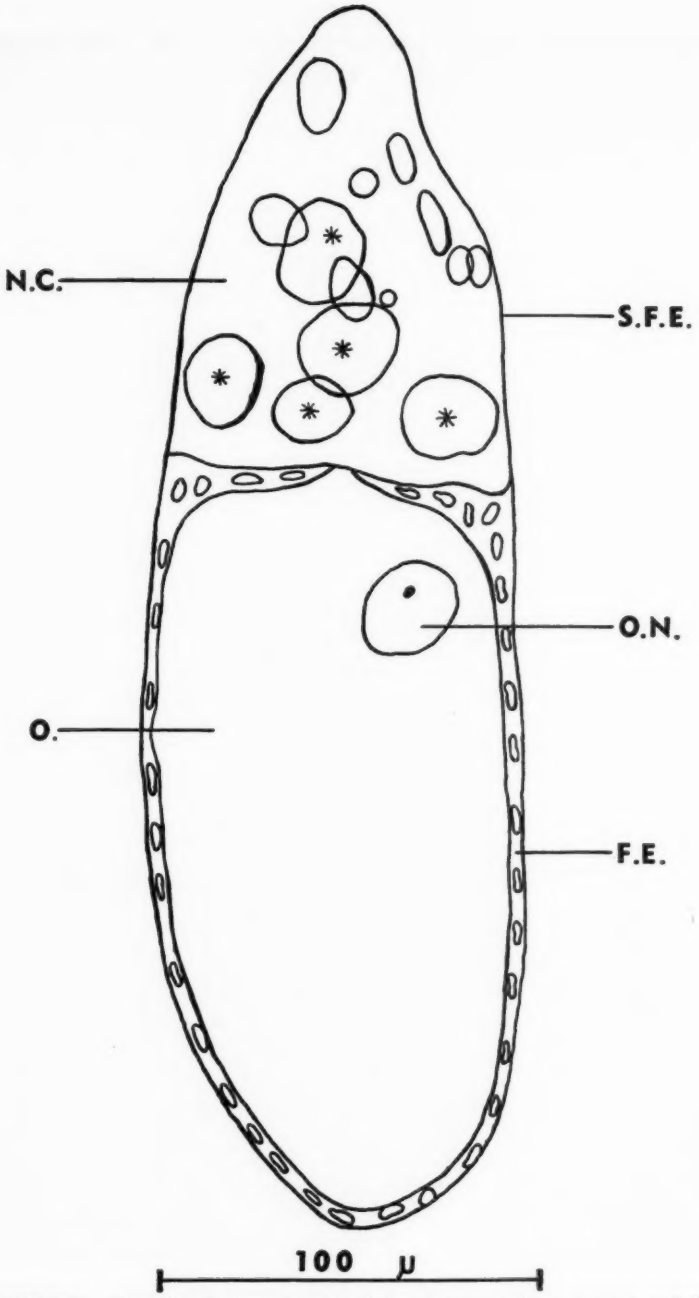


FIGURE 11. An early, stage 11 chamber. Fourteen nurse cells are present, and five contained banded, polytene chromosomes. The follicular epithelium (F.E.) has decreased in height.

Figures 7-12 show a series of representative yolky oocytes found in the ovaries of *fes* females from the 18° C. series. Because of shrinkage the dimensions of the chambers figured are 70-80% the true values. In the larger chambers the five nurse cell nuclei nearest the oocyte contain banded polytene chromosomes. In the outline drawings these nuclei are marked with asterisks. Nurse chambers containing subnormal and supernormal numbers of cells are commonly observed. The normal number of nurse cells is 15. It is obvious, however, that vitellogenesis proceeds with as few as 10 or as many as 30 nurse cells. That vitellogenesis can proceed normally when double the normal number of nurse cells are present had been shown previously (King, 1957b).

The border cells which play an important role in the formation of the micropylar apparatus (King, 1960) appear to make their normal migrations. The micropylar apparatus of the mature eggs studied appears to be normal.

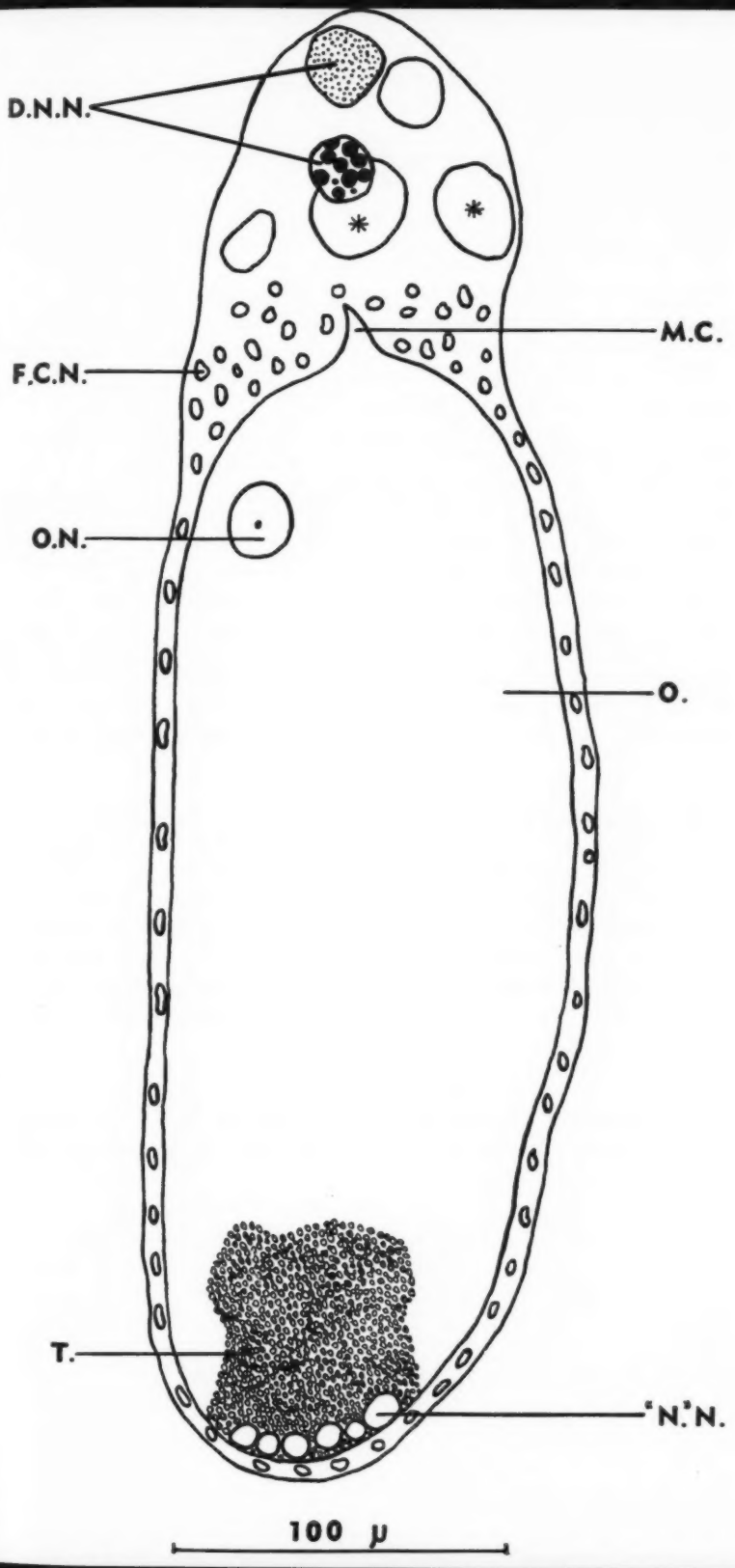
In only one instance was a *fes* female observed to lay mature eggs. The female in question was reared at 18° C. during preadult stages. During its adult life the fly was reared with wild type males at 25° C. On the tenth day of adult life two eggs were laid. These eggs bore misshapen appendages and failed to hatch.

Conclusions

It is interesting to note that in some tumorous chambers nurse-like cells are observed which are as large as those seen in stage 10 egg chambers and which show a similar nuclear morphology (see Figures 6, 9 and 10). Vitellogenesis is underway in stage 10 chambers, and the nurse cells play a vital role in this process (see King, 1960). Indeed, it is the nurse cells adjacent to the oocyte which show the most marked nuclear changes. However, the above observation demonstrates that the nurse cells can undergo similar morphological changes in the absence of an oocyte, but that yolk is not produced unless an oocyte is also present in the chamber.

The data presented demonstrate that within the ovarian tumors of *fes* females differentiation of cells into oocytes occurs at a much lower frequency than does nurse cell differentiation. The fact that nurse cell differentiation and oocyte differentiation are retarded to different degrees in this mutant suggests the mechanisms underlying

FIGURE 12. A late, stage 12 chamber. Six nurse cells are present, two of which contained degenerating nuclei (D.N.N.), and two of which contained banded, polytene chromosomes. F.C.N., follicle cell nuclei; M.C., micropylar cone. A posterior mass of tumorous tissue (T.) containing some nurse-like nuclei ("N." N.) is present in the oocyte.


D.N.N.
M.C.
F.C.N.
O.N.
O.
T.
"N."N.
100 μ

normal differentiation may be different and that the mechanism controlling oocyte differentiation may be the more complicated of the two. However, differentiation of nurse cells and oocytes occurs at a higher frequency at low than at high temperatures. However, low temperature seems to stimulate oocyte differentiation to a greater extent than nurse cell differentiation. Finally the data suggest that "nurse" cell differentiation in *fes* tumors occurs only during an early stage in the growth of the chamber.

In a previous paper (King, Sang and Leth, 1960) it was suggested that the plus allele of *fes* is responsible for the production of a substance which by initiating differentiation restricts cell division in the developing ovarian chamber. The *fes* gene is considered to be hypomorphic, and the system it controls operates best at low temperature in flies of certain ages and with certain residual genotypes.

Many mutant genes are known which produce more normal phenotypes at low than at high temperatures. In some cases this temperature sensitivity arises from the production of enzyme systems which function over a lower range of temperatures than those characteristic of the wild type system. The finding that *fes* is a temperature sensitive allele suggests that a similar mechanism may be operative here.

Summary

Hereditary ovarian tumors occur in females of *Drosophila melanogaster* homozygous for the second chromosomal, recessive gene *fes*. A cytological study was made of 16,000 chambers from *fes* females grown at 18, 21 or 25° C. It was found that within ovarian tumors differentiation of cells into oocytes occurs at a much lower frequency than does differentiation into nurse cells. However, differentiation of nurse cells and oocytes occurs at a higher frequency at low than at high temperatures demonstrating that *fes* is a temperature sensitive allele.

Acknowledgments

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FURTHER STUDIES ON THE PROTEIN COMPOSITION OF ALCOHOLIC EXTRACTS OF NEOPLASTIC RAT TISSUES*

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Previous work revealed marked differences between the chemical composition of alcoholic extracts of neoplastic tissues and that of similar extracts of normal muscle of inbred rats (25, 24, 15, 23). Non-dialyzable fractions prepared from these extracts also showed differences, both chemical and immunological (15). Electrophoresis data and ninhydrin reactions indicated that the tumor extracts contained several protein components which had no counterpart in the normal muscle preparations. Extracts from a sarcoma and a lymphoma were found to be antigenic for rabbits, whereas the materials extracted from the normal muscle tissue neither produced antibodies nor reacted with the antibodies to the tumor extracts.

The present paper deals with the properties of these non-dialyzable substances and the nature of their antigenic reactions. Since antigenicity is related to molecular size, the sedimentation behavior was studied in the analytical ultracentrifuge. To determine whether these substances are simple molecules or complexes containing carbohydrates and lipids, the staining reaction with dyes specific for protein-bound fat and carbohydrate was investigated. The precipitin reaction by agar gel diffusion was used for antigenic analysis. This technique gives a different line of precipitation for each antigenic component in a mixture; whereas the test tube precipitin test, previously used, does not really distinguish between multiple systems of antigens and antibodies.

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Materials and Methods

Source and Preparation of Materials

Most of the extracts used in these studies were prepared earlier in the laboratory of Dr. Florence Seibert, Henry Phipps Institute, Philadelphia, who kindly made this material available. Normal and tumor-bearing rats employed in preparation of the extracts were generously supplied by Dr. M. R. Lewis and Dr. P. M. Aptekman of the Wistar Institute, Philadelphia, and breeding stock was provided by Mr. A. L. Bates and Dr. Boland Hughes, formerly of the same Institute. We used only Wistar imbred rats implanted with Aptekman sarcoma No. 6, Lewis sarcomas No. 7 and No. 10, induced originally in this strain by methylcholanthrene injections, and Lewis lymphoma No. 8, which arose spontaneously in the same strain.

Details for preparing the extracts (25), including a slight modification of the original procedure (1, 2, 3), and for producing rabbit antisera to the extracts (15) have been previously described. These materials have been maintained in a freezer at —6° C. to —15° C., at which temperature the extracts remain in the liquid state due to their 30% alcohol content. The method used for fractionation of the extracts by dialysis and quantitative data concerning their protein concentration have been given elsewhere (15).

Determination of Protein Concentration of the Fractions

Protein analyses on the dialyzed fractions were carried out by two different procedures, (a) the determination of nitrogen by a modified Kjeldahl-Nessler method (16, 8) and calculation of protein concentration by the use of the standard protein factor of 6.25 and (b) the Lowry method (13) using bovine serum albumin as a reference protein.

Determination of Protein-Bound Carbohydrate and Fat

To determine whether any carbohydrate or fat was bound to the protein, samples of the dialyzed extracts were concentrated *in vacuo* to contain 10 to 20 mg protein per milliliter based on nitrogen analysis. Samples were then applied to Whatman No. 1 filter paper by the method used for paper electrophoresis experiments and dried in an oven at 60°C. The filter paper strips were then stained with Oil Red O(4) for lipids and by basic fuchsin (7) for protein-bound

carbohydrate. Chemical analyses were made for carbohydrate with the use of orcinol reagent in 70% H_2SO_4.

Analytical Ultracentrifuge Analyses

For analytical ultracentrifuge analyses*, samples of the dialyzed extracts were concentrated *in vacuo* to contain 10 mg protein per milliliter and dialyzed against pH 7.41 0.1 μ phosphate buffer. Analyses were made in a Spinco Model E centrifuge using a synthetic boundary cell (20).

Antigenic Analyses by Precipitin Tests in Agar Gel

Precipitin tests were carried out by the Ouchterlony agar plate diffusion method (19), as modified by Korngold (9). The antisera to the extracts were placed in the center cup, and antigen solutions, consisting of either whole extracts or fractions from the extracts prepared by dialysis, in the three or four surrounding cups. Initial tests were carried out at a series of 12 antigen dilutions ranging in concentration from 1,000 γ to 0.2 γ protein per milliliter based on nitrogen determinations. Plates were read after one week incubation at room temperature.

Removal of antibodies against components common to both sarcoma and lymphoma extracts from the antisarcoma extract antiserum was accomplished by absorbing the antiserum with the dialyzed lymphoma extract. This absorbed serum was then used in the agar plate diffusion test with the lymphoma and sarcoma extracts as antigens.

Results

Determination of Protein Concentration by Two Different Methods

Nitrogen determinations and analyses for protein by the Lowry method were carried out on the same preparations of the extracts which had been extensively dialyzed to remove the last traces of dialyzable nitrogen. Duplicate or triplicate analyses were made on two different extract preparations from each type of tissue. A comparison of the results obtained by the two different methods was made by calculating the ratio of protein based on Lowry determination to protein based on nitrogen determination, which is shown in Table I. The results show that the non-dialyzable material ex-

* Centrifuge analyses were carried out in the laboratory of Dr. Gail L. Miller, Pioneering Research Laboratory, Quartermaster Research and Engineering Center, Natick, Massachusetts.

TABLE I

COMPARISON OF PROTEIN VALUES OBTAINED BY NITROGEN DETERMINATION WITH THOSE OBTAINED BY LOWRY DETERMINATIONS ON THE NON-DIALYZABLE OR PROTEIN FRACTIONS OF SARCOMA, LYMPHOMA AND NORMAL TISSUE EXTRACTS

Non-Dialyzable or Protein Fraction Used	Ratio of $\frac{\text{Protein Based on Nitrogen Determination}}{\text{Protein Based on Lowry Determination}}$
Sarcoma No. 10 Extract	1.05
Lymphoma No. 8 Extract	1.09
Normal Tissue Extract	1.85

tracted from the tumor tissues gave almost identical results by both methods, while that from the normal muscle tissue gave only about one-half the value by the Lowry method as by the nitrogen method.

Determination of Protein-Bound Fat and Carbohydrate

The staining of proteins after electrophoresis on filter paper with stains specific for fat and carbohydrate presents a simple method of determining the presence of these substances associated with protein molecules and readily localizes them with various electrophoretic components in a mixture. The results of staining the dialyzed fractions with Oil Red O, specific for fat, and basic fuchsin, which stains protein-bound carbohydrate, showed that the proteins in both sarcoma and lymphoma extracts are simple protein molecules which bind neither fat nor carbohydrate. By the use of chemical analysis, using orcinol, the carbohydrate was located in the recovered and concentrated dialysates, while the dialyzed fraction gave negative results. Chemical analyses for fat were not repeated, since only traces had been found in the original extracts.

Analytical Ultracentrifuge Analyses

The sedimentation patterns for the non-dialyzable fractions of the sarcoma, lymphoma and normal muscle extracts are shown in Figure 1. The patterns of all three extracts show only a single component, and the sedimentation constants are not appreciably different, as seen from Table II.

TABLE II

SEDIMENTATION CONSTANTS OF THE NON-DIALYZABLE OR PROTEIN FRACTIONS FROM SARCOMA, LYMPHOMA AND NORMAL MUSCLE TISSUE EXTRACTS

Non-Dialyzable or Protein Fraction Used	Sedimentation Constant $S \times 10^{-13}$
Sarcoma No. 10 Extract	0.746
Lymphoma No. 8 Extract	0.728
Normal Muscle Tissue Extract	0.685

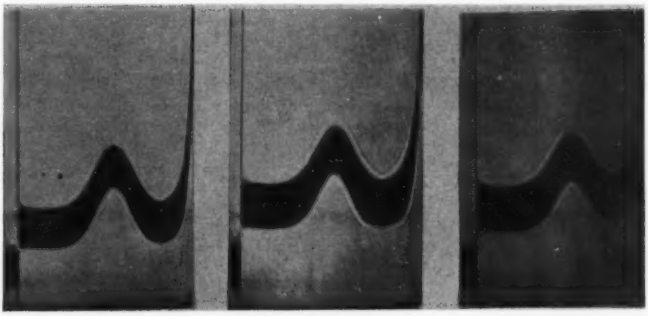

FIGURE 1. Sedimentation patterns from analytical centrifuge analyses of non-dialyzable fractions of alcoholic extracts of (left to right) sarcoma, lymphoma, and normal muscle tissues. Phosphate buffer, pH 7.34, 0.1 ionic strength.

Antigenic Analyses by Precipitin Tests in Agar Gel

The precipitin reaction on agar diffusion plates between the extracts and their respective antisera showed that two antigenic components are common to both the sarcoma and lymphoma extracts, while a third one is found only in the sarcoma extract. The normal muscle tissue extract did not react with antiserum to either tumor extract, and the serum from rabbits injected with the normal muscle extract did not give any reaction with the normal muscle extract itself or either of the two tumor extracts. The precipitin reaction between antisarcoma extract antiserum and the sarcoma, lymphoma and normal muscle extracts used as antigens is shown in Figure 2. Two lines about half-way between the center and outer cups were obtained with both the sarcoma and lymphoma extracts, while a third line close to the center cup was obtained only with the sarcoma extract. The normal muscle extract gave no reaction.

The reaction shown in Figure 2 was obtained with antigen concentrations of 50 γ protein per milliliter of antigen solution, although other tests were made with antigen concentrations ranging from 1,000 γ to 0.5 γ protein. At an antigen level of 5 γ and below, no lines were obtained. At the higher antigen concentrations, resolutions of the two components common to both tumor extracts which are close together, was not always accomplished, but they appeared as a single line of precipitation. The line of precipitation found only with the

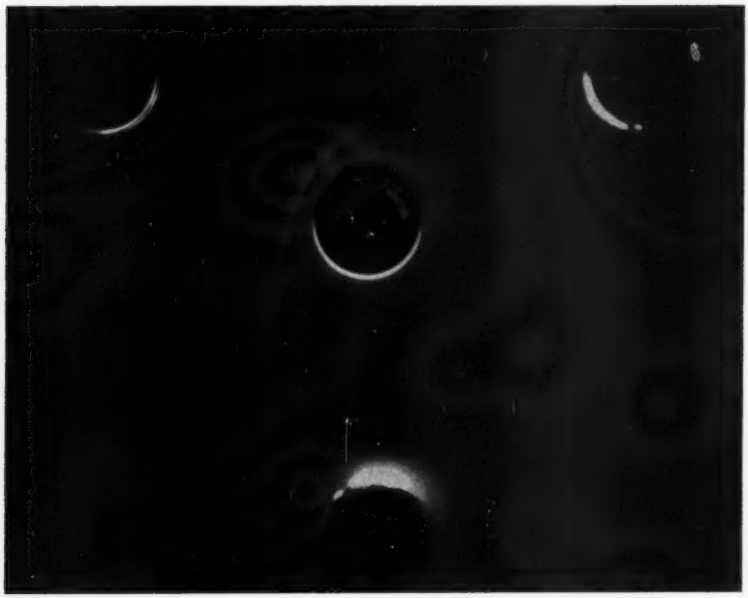

FIGURE 2. Precipitin reaction on Ouchterlony agar plate between the three extracts and antiserum to the sarcoma extract. Center cup: antiserum to sarcoma extract; left-hand cup: sarcoma extract; right-hand cup: lymphoma extract; bottom cup: normal muscle extract. Antiserum, undiluted; antigen concentrations, 50 γ protein per milliliter.

sarcoma extract and its antiserum, the heavy line closest to the center cup, was obtained over an antigen concentration range of 200 γ to 20 γ protein per milliliter.

When the sarcoma extract was used as an antigen with its own antiserum, three lines of precipitation were formed, but, when it was used with antilymphoma extract antiserum, only two were obtained. When the lymphoma extract was used as antigen on the same plates with either its own antiserum or antiserum to the sarcoma extract, only two lines of precipitation were obtained, which shows that this third antigenic component is not present in the lymphoma extract and that antibodies to this component do not cross react with any substance in the lymphoma extract. The antigenic component charac-

teristic of the sarcoma extract has been found in six out of seven different extract preparations from sarcomas No. 6, No. 7 and No. 10. Five different extracts from lymphoma No. 8 were tested with anti-sarcoma extract antiserum and the component characteristic of the sarcoma extract was found in none of them.

Other experiments have shown that the proteins in the tumor extracts are responsible for these antigenic reactions. With the use of the non-dialyzable fractions from the tumor extracts, the same lines of precipitation were obtained. Absorption of the antiserum to the sarcoma extract with the dialyzed and concentrated lymphoma extract removed completely the antibodies to the two components which are common to both tumor extracts. The antiserum thus absorbed, when used on the agar plates with the sarcoma extract as an antigen, still gave the heavy line of precipitation close to the center cup which is characteristic of the reaction between the sarcoma extract and its antiserum.

In addition to normal muscle preparations, extracts similarly prepared from other normal rat tissues, and tissues from tumor-bearing animals, as well as normal rat blood, have also been used as antigens at four different dilutions. Normal rat blood, and extracts from spleen, kidney, and lung of both normal and tumor-bearing animals did not react at all. Rat liver extracts gave diffuse lines at the highest concentrations used, a reaction which was also obtained when normal rabbit serum was used instead of antisera to the tumor extracts. These diffuse lines of precipitation did not correspond in any way to the lines of precipitation obtained between the tumor extracts and their antisera. The lines formed by the liver extracts did not join those formed by the tumor extracts which showed that they were due to entirely different antigens. The tumor extracts gave no lines with normal rabbit serum.

Discussion

The most striking difference revealed by the present work concerning the nature of the protein or non-dialyzable fractions of alcoholic extracts of tumors and normal tissues is that the sarcoma extract contains an antigenic component not present in the lymphoma extract. Two additional components in the sarcoma extract are also found in the lymphoma extract. These findings no doubt reflect the differences observed previously by paper electrophoresis (15), which indicated

that the non-dialyzable fraction of the sarcoma extract contained three electrophoretic components of similar mobilities, whereas only two of these appeared to be in the corresponding fraction of the lymphoma extract. The absence of fat and carbohydrate in these non-dialyzable fractions leaves little doubt that these components are protein in nature.

Normal muscle extract gives no lines of precipitation on the Ouchterlony agar plate either with its own antiserum or antisera to the two tumor extracts. This finding confirms previous work with the test tube precipitin test (15), which showed that the normal rat muscle extract did not produce antibodies in rabbits. This does not mean that normal muscle tissue does not contain antigenic proteins, but simply that this alcoholic extraction procedure removes antigenic proteins from tumor tissue, but not from muscle. In this respect, the results of these experiments differ from those of others using different methods and types of extractions, who have found various normal tissues to be antigenic and react with tumor antisera (9, 14, 29).

The question as to whether any normal tissue serves as a proper control for studying tumor tissues has been the subject of much discussion (26, 27, 28, 17). Comparative studies on the amino acids (21, 22, 24), as well as the enzymes (14, 5), isolated from various normal and tumor tissues have shown that, with respect to these components, different types of tumor tissues are more alike than different types of normal tissues. In the present studies none of the extracts from the five different normal tissues from inbred rats reacted with antitumor antibodies, suggesting that the proteins which produced these antibodies are specific for the tumor tissue. It appears, also, that with respect to the tumor proteins themselves, the two tumor extracts contain two identical protein components; yet, in addition, the sarcoma has one protein which appears to be characteristic of this type of tissue only. The fact that the two lines of precipitation formed on the Ouchterlony plates by both tumor extracts join each other at the corners indicates that they are identical and not cross-reacting antigens (19).

Further evidence has been obtained for the marked difference in the substances extracted from the tumor and normal muscle tissues by making protein determinations by two different methods. The

normal muscle extract gives almost twice the protein value when based on nitrogen determinations as it does when based on Lowry determinations, which recalls previous findings (15) on the differences in ninhydrin-color producing values of the normal and tumor tissue extracts.

Some of the factors responsible for antigenicity have been discussed by Landsteiner (10). One of the most striking of these is the correlation of molecular size with antibody producing ability. The similarity of the sedimentation rates of the three preparations suggests that the molecular size of the non-dialyzable fraction of the muscle extract was not sufficiently different from that of the tumor extracts to account for its lack of antigenicity.

Neither can the antigenicity of the tumor extracts be explained on the basis of protein-carbohydrate-lipid complexes, since no bound fat or carbohydrate was found in any of the dialyzed fractions. This eliminates the possibility suggested by Hauschka (6) that lipoprotein antigens may be responsible for the immunizing properties of these particular extracts.

The fact that the sarcoma extract has been found to contain an antigenic protein which is not in lymphoma extract raises the question as to what the relationship of these proteins may be to tumor-host resistance. The lymphoma is a very malignant tumor which kills the host in 9-15 days, whereas survival time is much longer for the sarcomas. Work is now in progress on the isolation of this component in sufficient quantities to test for immunizing activity in animals.

Other differences in the two types of tumors may also be associated with the presence of this protein in the extracts from the sarcoma tissue and its absence in extracts prepared from lymphoma tissue. For example, the lack of this protein may in some way be related to the fact that styrylquinoline dye therapy induces complete regression of the lymphoma but not of the sarcoma (11). Another interesting difference between the tumors concerns their origin. Although all are now carried by transplantation, the lymphoma arose spontaneously in the inbred strain of rats, whereas the sarcomas were induced by methylcholanthrene. It seems possible, therefore, that the protein found only in the sarcoma extracts may be characteristic of carcinogen-induced tumors. These questions can be answered only by further studies on other types of tumor tissues.

Summary

Further studies have brought out interesting differences between the protein or non-dialyzable fractions of alcoholic extracts of a lymphoma, sarcomas and normal muscle tissue of inbred rats. Antigenic analysis by the agar plate diffusion technique revealed two antigenic components common to the non-dialyzable fraction of both tumor extracts and a third component present only in the sarcoma extract. Extracts of muscle, spleen, kidney, lung and liver of normal and tumor-bearing inbred rats and rat plasma did not react with antibodies to any of these three components. Non-dialyzable material extracted from the normal muscle tissue yielded only about one-half the protein value by Lowry determination as by nitrogen analysis, while both tumor tissues gave similar values by both methods, which was further evidence for a difference in the nature of material extracted from the tumor and normal muscle tissue. The antigenicity of the tumor extracts did not appear to be related to molecular size or the presence of lipid or carbohydrate in the preparations.

Acknowledgment

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Growth, 1961, **25**, 79-87.

REGENERATION OF CELL STRUCTURE AND RESTORATION OF PROTEOLYTIC ACTIVITY FOLLOWING ETHIONINE FEEDING IN RATS

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INTRODUCTION

The antimetabolite of methionine, ethionine, can cause considerable alterations in the cytology and functioning of the pancreatic acinar tissue (Alvizouri and Warren, 1954; De Almeida and Grossman, 1952; Farber and Popper, 1950; Goldberg *et al.*, 1950; Goldberg and Chaikoff, 1951; Loring and Hartley, 1955). It was first reported that ethionine destroyed the cytology of the acinar tissue but it was later shown that regeneration of this tissue could take place (Edwards and Brehme, 1959; Fitzgerald, 1958; Fitzgerald and Alvizouri, 1952; Kinney *et al.*, 1955). Since it has been shown in this laboratory that acinar-cell-free (duct-ligated) rats were unable to grow properly, (Edwards and Brehme, 1956) a study of pancreatic deficiency, produced by ethionine feeding, and body growth was in order. As a measure of pancreatic function, the potential tryptic activity of the pancreas was estimated. Since ethionine gives us a fairly uniform and gradual destruction of the acinar tissue of the pancreas, it was hoped that the the proteolytic activity of the pancreas could be reduced to a level where no growth would be obtained after the ethionine was removed from the diet.

MATERIALS AND METHODS

Young male rats weighing about 100 gms. were placed on a diet containing 0.5% dl-ethionine made up in Purina Chow and were allowed to eat ad libitum for three weeks. After this period some rats were fed only Purina Chow while others were fed a 1% 3× pancreatin diet made up in Purina Chow. Some rats from each group were sacrificed after the initial ethionine feeding and some were

sacrificed at two weeks, others at four weeks and some at six weeks following the ethionine feeding. Control rats were fed only Purina Chow and were sacrificed at the same time intervals as were the experimental animals. Prior to killing, the animals were fasted 24 hours. Some of the ethionine-fed animals died in this fasting period immediately following the ethionine feeding and could not be used in this study.

At the time of sacrifice the entire pancreas or pancreatic area was removed. It was first extracted of all its fat and then dried and weighed. The glycerine extract was used for analysis. The method of Kunitz (1946-1947) was modified to analyze for trypsinogen. Trypsinogen was activated by enterokinase prepared from hog intestine (Sumner and Somers, 1953). Casein was used as a substrate. The soluble protein products were read on a spectrophotometer at a wave length of 280 mu. A trypsinogen standard was used for each set of analyses. All samples were run in triplicate. The data have been converted to mg. of trypsinogen.

Results

Cytology

In Figure 1 is shown the apparently complete destruction of the acinar tissue of the pancreas. The same effect has been reported by several investigators (Alvizouri and Warren, 1954; De Almeida and Grossman, 1952; Farber and Popper, 1950; Goldberg *et al.*, 1950; Goldberg and Chaikoff, 1951; Loring and Hartley, 1955). After two weeks the pancreatic acinar tissue has recovered from the ethionine insult (Figure 3) and looks architecturally identical to the normal pancreas seen in Figure 4. Other investigators (Fitzgerald, 1958; Fitzgerald and Alvizouri, 1952) have shown some of this regeneration of the pancreatic tissue under these same conditions.

Growth

In Table I it can be shown that the ethionine-fed rats begin to grow on discontinuing the ethionine in the diet. It should be mentioned that an occasional rat is not able to grow because of intestinal bleeding. None of the rats with known intestinal bleeding have been included in this study.

Not only is growth very rapid in its onset but the growth rate on

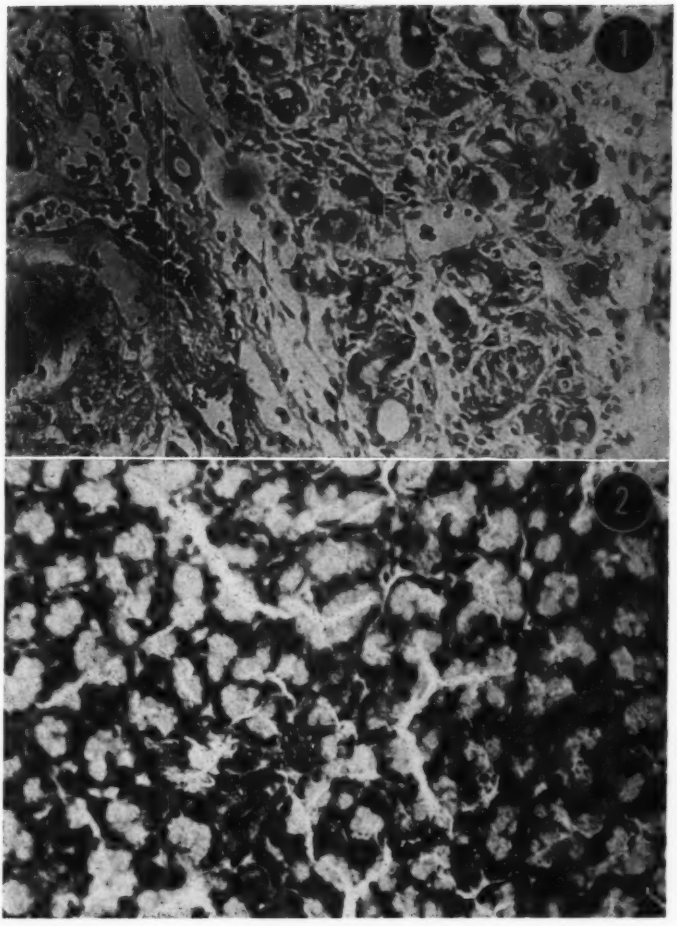

FIGURE 1
Section of the rat pancreas showing acinar cell destruction after feeding 0.5% ethionine in the diet. Stained with hematoxylin and eosin. 715 ×.

FIGURE 2
Section of the normal rat pancreas stained with hematoxylin and eosin. 715 ×.

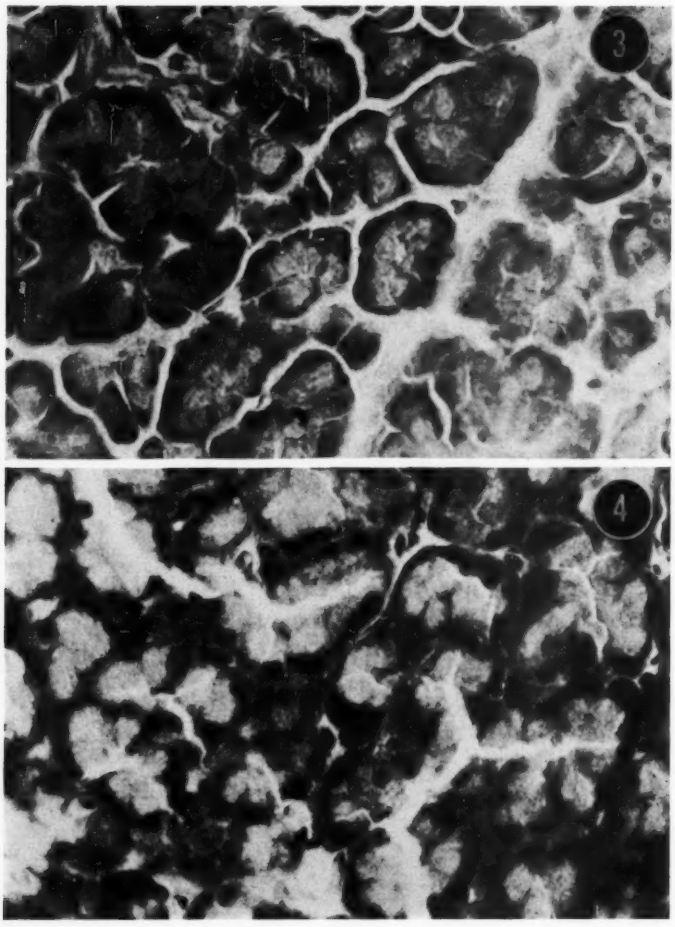

FIGURE 3
Section of the rat pancreas showing acinar cell regeneration two weeks subsequent to termination of the ethionine diet. Stained with hematoxylin and eosin. 1250 ×.

FIGURE 4
Section of the normal rat pancreas stained with hematoxylin and eosin. 1250 ×.

TABLE I

N	Rat Group	Wt. Gain (gms/day)	
31	A	5.73 ± 1.55*	
32	B	5.31 ± 1.93*	
26	C	4.31 ± 1.08*	
		Mean difference	P
Group A vs. Group C		1.42	< .01
Group B vs. Group C		1.00	< .02
Group A vs. Group B		.42	< 0.4

N Number of animals.
* Standard deviations.
A Rats fed 1% 3 × pancreatin diet following ethionine.
B Rats fed no pancreatin diet following ethionine.
C Untreated control rats.

the pancreatin diet is increased 33% and on the no pancreatin diet 23% above that of the normal rat of the same age. Since growth in these animals represents nearly a linear function of time for the first two weeks following the ethionine treatment, the average weight gain per day is compared rather than the growth index (K) as described in a previous paper (Edwards and Brehme, 1960).

The trypsinogen levels in mg. per gram of body weight have been plotted for the various periods of analysis (Figure 5). In the early periods following the ethionine feeding, the trypsinogen concentration is very low in the pancreas but gradually increases after that to a point where it approaches the normal level. The normal level is approximately reached six weeks following the ethionine feeding.

Still another way of plotting the data is shown in Figure 6. This figure illustrates that the total trypsinogen reaches a plateau at a certain weight of the animal. This level remains approximately constant even though the rat continues to gain weight. The experimental animal approaches this level after six weeks following ethionine feeding.

Discussion

Ethionine, although it destroys the cytology of the pancreatic acinar tissue, does not produce a permanent damage to the cell. These cells are able to regenerate or to re-establish their original cytological appearance. The potential tryptic activity does not keep pace with this recovery or perhaps the cells are slow in developing their normal secretory function. Pancreatin has very little if any influence on this recovery or regeneration.

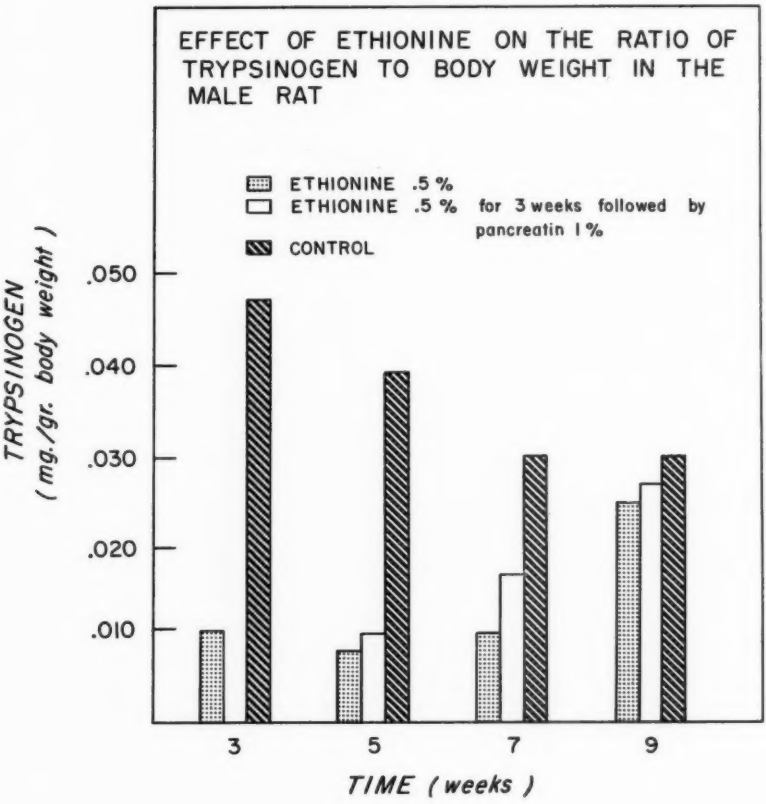
EFFECT OF ETHIONINE ON THE RATIO OF TRYPSINOGEN TO BODY WEIGHT IN THE MALE RAT
ETHIONINE .5 %
ETHIONINE .5 % for 3 weeks followed by pancreatin 1 %
CONTROL
TRYPSINOGEN (mg./gr. body weight)
.050
.040
.030
.020
.010
3
5
7
9
TIME (weeks)

FIGURE 5

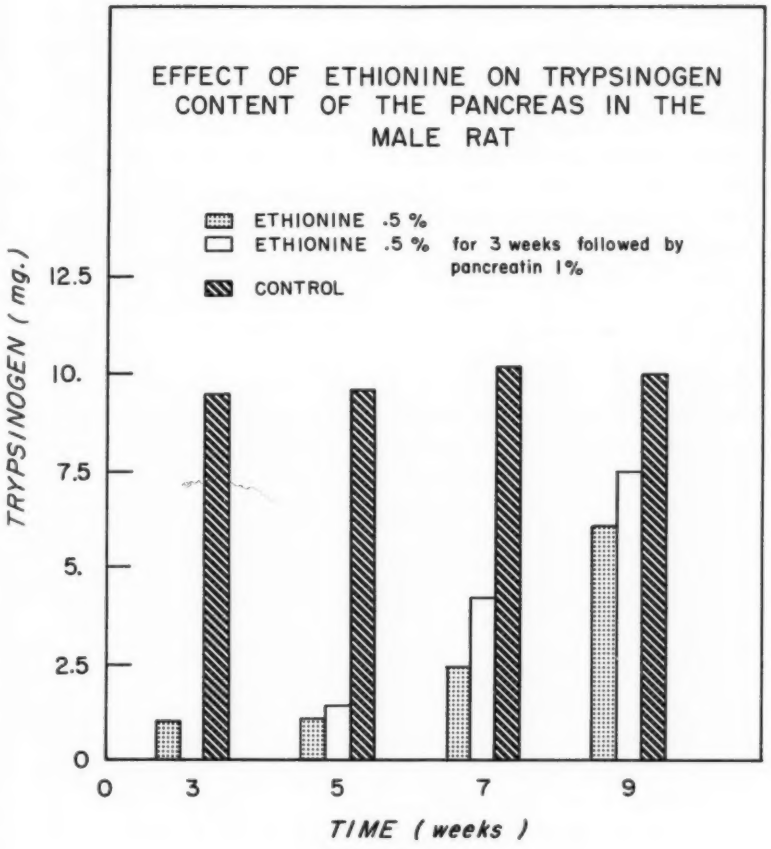
EFFECT OF ETHIONINE ON TRYPSINOGEN CONTENT OF THE PANCREAS IN THE MALE RAT
ETHIONINE .5 %
ETHIONINE .5 % for 3 weeks followed by pancreatin 1%
CONTROL
TRYPSINOGEN (mg.)
12.5
10.
7.5
5.
2.5
0
0
3
5
7
9
TIME (weeks)

FIGURE 6

Ethionine acting as an antimetabolite of methionine has two actions on protein metabolism. First it interferes with the synthesis of the proteolytic enzymes of the pancreas (Kroboth and Hallenbeck, 1954; Sidransky and Farber, 1956) which in turn inhibit the breakdown of protein in the gut. Secondly it interferes with the synthesis of protein elsewhere in the body (Farber and Corban, 1958; Pool and Spaet, 1954; Simpson *et al.*, 1950; Wang *et al.*, 1958). Our data suggest that its effects on growth are related to protein synthesis more than on protein breakdown in the gut. This study supports this view since excellent growth is obtained when tryptic activity has not improved.

One can readily see that we have only scratched the surface of the growth problem involved. From this study we see that rats can grow with a pancreas that has considerable damage done to it, whereas in the duct-ligated rats described in an earlier paper (Edwards and Brehme, 1956) no growth is obtained when no acinar tissue is present. It should be pointed out, however, that in the duct-ligated rats only a small piece of pancreas is necessary for growth. Thus it appears that only a minute amount of active pancreas or, as in the case of the ethionine treated rats, many small fragments of pancreatic cells are necessary for growth.

We are unable to explain the increased growth rate seen in the early stages of regeneration.

Summary

Ethionine, although it seems to destroy the cytology of the acinar tissue, does not produce a permanent damage.

Regeneration of acinar tissue is rapid after ethionine feeding but recovery of the potential proteolytic activity is greatly delayed.

The ethionine treated pancreatic tissue still permits growth after the removal of the ethionine.

This growth is greater than that found in the control rats of the same age.

The critical growth regulating mechanism of the acinar tissue of the pancreas is still unknown.

Acknowledgments

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Growth, 1961, **25**, 89-106.

OBSERVATIONS ON DEFICIENT CHROMOSOME GROUPS IN DEVELOPING SCIARA LARVAE

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INTRODUCTION

The present paper deals with a series of aberrant phenomena encountered in a study made years ago for another purpose. Because the data apparently reflect fortuitous events which could not be induced deliberately they are recorded here for possible use by other investigators. They appear to have significant bearing on problems of growth and development. No attempt will be made to deal with the broad subjects involved or the literature bearing on them, although a few comments will be made on some special points.

The earlier study was part of the cytogenetic investigations in our laboratory on "fungus gnats" of the genus Sciara. It involved examination of the chromosome groups in the germ line in embryos and young larvae (Metz and Schmuck, 1931). In addition to the expected normal conditions, cases were found in which strikingly deficient chromosome groups were present in cells which appeared normal in most other respects, including the capacity for mitosis, growth and multiplication. The main interest of the phenomena, perhaps, lies in the additional light they may throw on the extent to which cells of higher animals are able to function with such incomplete and unbalanced sets of chromosomes. Another reason for recording the data is the possibility that a mutant gene is instrumental in inducing the loss of chromosomes.

The species dealt with is *Sciara coprophila* Lintner, a species used extensively in the earlier studies just referred to. In one of these DuBois (1933) recorded deficient chromosome groups in somatic nuclei found in cleavage stages in eggs. Our observations serve to

* The earlier studies which provided the material for the present work were carried out mainly in the Department of Embryology of the Carnegie Institution of Washington.

extend those of DuBois to a later stage of development and to gonad cells, which present different aspects.

Inasmuch as the present data come from a series of sporadic cases, most of which require individual examination, several points should be made clear at the outset in order to avoid undue repetition. These deal mainly with the nature of the study and the methods of presentation of the data.

With the exception of a small amount of supplementary information at the end, the study is based on examination of early larval ovaries. At this stage the gonads are sac-like and normally contain only cells of the germ line (Berry 1941). The data are from the germ line cells. The original study involved many other ovaries of comparable age in which no suitable mitotic figures, or only normal chromosome groups, were found. The present study deals only with those exhibiting deficient groups. At best only a few mitotic figures are found in any one gonad, and rarely are any mitoses with normal chromosome groups found in an ovary exhibiting mitoses with deficient groups. In some cases, however, interphase nuclei with "prochromosomes" can be identified as having the full, normal number. In all cases the cells identified as having deficient groups are in compact clusters in which all the cells of any one cluster appear to have the same deficient group of chromosomes. In each case the cells are in mitosis, exhibiting essentially synchronous mitotic behavior. Since we are dealing with sporadic phenomena, we have no choice but to use such mitotic figures as are available where they permit counts or reliable estimates of chromosome numbers. Frequently this requires using pro-metaphase or early metaphase stages, before the chromosomes become fully oriented or in one plane. Consequently, as will be noted below, exact details of length and shape of some chromosomes are not reflected in the accompanying drawings.

Owing to use of the Feulgen method, which tends to color only the chromosomes, the present material is not satisfactory for study of ordinary cytoplasmic constituents, and in some cases is not reliable for detection of cell boundaries or cell sizes. Hence, comparative cell sizes within a gonad are only reported where they seem distinct and significant. When comparison can be made between normal and deficient cells there seems in general to be a rough correspondence between cell size and chromosome number within a gonad. No definite

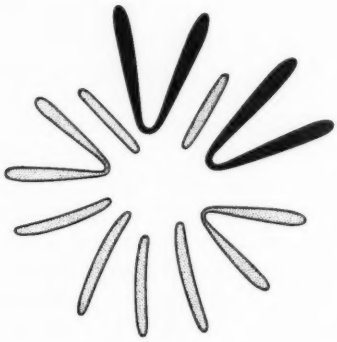
generalization can be made here, because in each case most of the cells in the ovary are in interphase and there is no certainty as to how many are normal and how many deficient. Ordinarily deficient cells can only be identified when in mitosis.

In dealing with Sciara it is difficult to make an account of the present kind both clear and concise, because in this genus many aspects of chromosome behavior and genetic behavior deviate widely from those in more orthodox or "standard" organisms; and without an understanding of these deviations most observations would have little meaning. It is necessary, therefore, to preface the present report with an outline of the problems to be considered and the bearing of normal Sciara phenomena upon them. For background details the reader is referred to earlier papers such as those cited below in the text, including the general review (Metz, 1938).

(1) It should be emphasized first that in Sciara certain chromosome-eliminating events occur regularly as normal processes of reproduction and development in both germ line and soma. These normal processes should not be confused with the phenomena under present consideration, although they may help to throw light on the latter. (2) The deficient chromosome groups we are dealing with contain fewer chromosomes than are normally present in the material involved, and in most cases fewer than in any diploid cells here. (3) In Sciara both sexes possess two X chromosomes in the germ line; no Y chromosome is present. Furthermore, the sperm regularly transmits two X chromosomes and the egg one, giving the zygote three. Compensating elimination processes which occur regularly in both sexes serve to maintain an equilibrium and to effect proper sex determination. (4) Normally and consistently two radically different mechanisms of chromosome elimination are involved. One operates at mitosis, the other during interphase. The former is found in normal eliminations from the somatic line during cleavage; the latter serves in both sexes to remove the surplus X chromosome from the germ line just before the gonads are formed. (5) In addition to sex chromosomes and ordinary autosomes *Sciara coprophila* possesses a large "limited" chromosome, so named because it is regularly eliminated from the soma during early cleavage and hence is, in effect, limited to the germ line. It is relatively inert genetically and may be present in duplicate or triplicate without noticeable effect on the

fly. In the oogonial cells dealt with here either one or two should be present. (6) In the germ line cells of Sciara, during early development, the chromosomes may be counted, not only at mitosis, but during part of the interphase stage where each forms a compact body or prochromosome. Such counts will be referred to below.

The normal chromosome group to be expected in the gonad cells described here is shown in Text figure *A*. It consists of nine or ten chromosomes of three morphological types: 1 pair of rod-like X chromosomes and 2 pairs of rod-like autosomes, all approximately the same size; 1 pair of V-shaped autosomes; and 1 or 2 larger V-shaped "limited" chromosomes.



TEXT FIGURE *A*

Diagram of normal oogonial or spermatogonial chromosome group found in young larvae of *Sciara coprophila* Lintner. As explained in the text, there may be either one or two of the large V-shaped "limited" chromosomes (shown in solid black). These are relatively inert genetically. The remainder of the group consists of a pair of rod-like X chromosomes, two pairs of rod-like autosomes and a pair of small V-shaped autosomes.

We are indebted to Dr. Donald F. Poulson for his kindness in reading the manuscript of this paper and making valuable suggestions.

Material and Methods

The gonads used in the present study are almost all ovaries. They were derived from matings within our laboratory stock, number 4941, which was used to maintain the recessive mutant "swollen" (symbol *sw*). This stock was originally derived from a cross between a wild

type stock from Anaheim, California and an eastern stock carrying *sw*. One question which arises is whether the gene for "swollen" or something in the Anaheim stock may have had an influence on the induction of the deficient chromosome groups.

Very young larvae were used, mostly not over four days after emergence from the eggs. The gonads were dissected out, or exposed by cutting off the anterior part of the larva, and fixed immediately in strong Flemming's fluid or Bouin's. All the staining was done by the Feulgen method so that studies could be made on whole mounts, without sectioning. About a third of the present material represents whole mounts, the remainder serial sections. Since the gonads are very small, fixation was practically instantaneous and gave good results.

In the original study more than 200 slides were prepared. A little more than half of these either failed to show any germ line cells in mitosis, or had none suitable for study. A total of 101 are recorded as suitable for determining chromosome number in one or more cells. Among these, 7 exhibit deficient chromosome groups (in a total of 8 ovaries) and two others also appear to have deficient groups.

Supplementing the observations just referred to, some notes will be added on mitotic figures in somatic cells from these same specimens and also those in an entire egg, from another source, which exhibits deficient groups in cleavage mitoses.

Deficient Chromosome Groups in Gonads

The general nature of the evidence here may be seen by comparing the figures in Plate 1 with the expected chromosome group shown in Text figure *A*. Whereas 9 chromosomes (or 10) are expected, the deficient groups range from 7 down to 4 and probably to 3. Numerous groups of 4 and 5 are found. Before examining individual cases a few points of special significance may be recalled. The first is that the deficient chromosome groups are in clusters, each with similar groups. In other words, it seems clear that all the cells in the cluster have the same deficient number and have been derived through mitotic multiplication of an ancestral deficient cell. Except for chromosome number, and in some cases a difference in size, nothing has been observed to distinguish the deficient cells from their ordinary neighbors in the gonad. The fate of their descendants

is not known, of course, but up to the stage dealt with here the deficient cells appear to have functioned as well as their associates. Owing to the technique used (Feulgen method) no cytoplasmic details are evident.

Six of the drawings (Figs. 3, 4, 7, 11, 12, 13) are from cells in unsectioned total mounts; the remainder (1, 2, 5, 6, 8, 9, 10) are from sectioned material. For the most part the latter are as reliable as the former, because it is usually clear that the cell in question is not cut and, more importantly, the same deficient group has usually been detected in two or more cells of the cluster. The drawings are made from polar views of figures in pro-metaphase, metaphase and beginning anaphase stages. In some cases, as mentioned above, individual chromosomes appear foreshortened because they are slanted vertically, and rod-shaped members of equal actual length may appear different. Where they have no significance in the present connection, such features will be ignored. Most of the material is included in the descriptions given below. The latter are arranged in sequence and include in each case pertinent accessory information as well as a reference to the appropriate drawings and slide number.

Digest of Data Presented in Descriptive Portion

For readers who may not be interested in the details, a general picture of the chromosomal phenomena in the cases represented by figures 1-13 may be outlined as follows: Eight ovaries are involved. The chromosome numbers in the respective cases are 7, 6 (or 7?), 7, 6, 5, 5, 5 (or 4?) and 4. (A case which may involve only 3 is described but not figured.) Apparently the large "limited" chromosome is missing in each case. The other missing chromosomes tend to be rods. The two autosomal V's are, or may be, present in all but the last case cited (which may represent a haploid group). The number of cells per deficient cluster appears to range from about 12 or 15 to 20 except in the last and second from last cases cited (slides 2886-III and 2885-V). Morphogenetic and related aspects are dealt with in the descriptions below.

Figs. 1, 2 and 5:* slide 2902-III; ovaries; sections. Figures 1 and 2 are from adjacent cells in a cluster of approximately 16 oogonia in mitosis, all apparently having the same group of 7 chromosomes, consisting of the 2 autosomal V's and 5 rods. The V's are seen at the upper left in Fig. 1 and at the left in Fig. 2. The V-like structure at the right in Fig. 2 is two rods, overlapping at one end. In another ovary on this same slide is another cluster, estimated at approximately 16 oogonia, in mitosis. Fig. 5 is drawn from one of these. It has either 6 or 7 chromosomes. Other figures in this cluster appear to have the same group, but are not suitable for accurate analysis. These two ovaries are probably from the same larva. In

* All figure numbers refer to drawings in Plate I.

both cases the cell walls are distinct and the deficient cells appear to be as large as the normal ones. In the first case the cluster of deficient cells is near one end of the ovary, in the second case the cluster is near the middle of the ovary, with one margin near the periphery. In each case the contour of the ovary is smooth, with no evidence of distortion in the region of the deficient cluster.

Figs. 3 and 4: slide 2884-VII; ovary; whole mount. These are from neighboring cells in a very small ovary consisting of an estimated 60-70 cells. Four cells are in a late prophase or early metaphase stage, before the chromosomes are in metaphase alignment; hence shapes and sizes are not accurate. Both figures (3 and 4) appear clearly to have 7 chromosomes. Although not evident in the drawings, they seem to be 2 small V's and 5 rods. Some of the other cells in this gonad may have eight or nine chromosomes, but they are not in a stage suitable for accurate counting.

Figs. 6 and 8: slide 2908-IV; ovary; sections. These two figures are from cells in a cluster estimated to contain between 15 and 20 cells, all in mitosis. Six figures, in addition to those shown here, also have 6 chromosomes and some others appear to have 6. No figures with larger numbers could be detected. The chromosome group shown in Fig. 6 is a flat metaphase plate consisting of the two small V's and four rods. It fills the equator of the spindle completely. Neither in this nor in the one shown in Fig. 8 is there any gap to indicate that the figure is cut. The cluster of dividing cells here is estimated to represent perhaps one sixth of the gonad in bulk. The cells in this cluster appear to be fully as large as the others in the gonad. The latter are in an interphase stage which does not permit counting the chromosomes. The ovary here is small and is sectioned transversely. The cluster of deficient cells in the cross section is shaped like a piece of pie extending from the periphery to about the center of the section. No evidence is seen of any distortion of the periphery of the gonad by the deficient cluster.

Fig. 10: slide 2901-I; ovary; sections. This figure is from one of a cluster of perhaps a dozen dividing small cells in approximately the middle of the ovary. The other cells of the ovary are much larger but are in an interphase stage which does not reveal the chromosomes. In the cluster are four figures suitable for counting. Each has 5 chromosomes, and all appear to have the group shown here—2 small V's and 3 rods.

PLATE I

Deficient chromosome groups in *Sciara coprophila* Lintner. For additional details see text. Although the magnification is the same in all drawings the cell boundaries indicated here do not necessarily reflect the true cell sizes. Figs. 1-13, from germ line cells in ovaries of very young larvae. Fig. 14, from a somatic cell in a very young larva.

1, 2, from one ovary, each with 2 autosomal V's and 5 rods; the V's are at the upper left in Fig. 1 and at left in Fig. 2; the V-like structure at right in Fig. 2 is two rods, overlapping at one end. 3, 4, from one ovary, apparently 2 V's and 5 rods in each; in 3, two chromosomes at upper right overlap to make the M-shaped structure; see text for details. 5, consists of either 6 or 7 chromosomes—4 rods, 1 small V and, at top, what looks like a V but may be 2 rods touching at their inner ends. 6, two small V's and 4 rods. 7, apparently the V-shaped structure in lower part of figure is composed of 2 rods which overlap at their inner ends to simulate a V, making a total of 5 chromosomes. 8, six chromosomes, apparently 2 V's and 4 rods. 9, group consisting of 5 or 4 chromosomes; the structure at the lower left could be either a single V or two overlapping chromosomes; presumably the member at the right is not a V with the apex pointing outward, but is a rod not yet oriented. 10, consists of 2 V's and 3 rods. 11, 12, 13, from one ovary; group consists of 1 small V and 3 rods, may be haploid group. 14, somatic group, flat metaphase plate lacking one member of the autosomal V-shaped pair of chromosomes. Camera lucida drawings by Miss Louise H. Buck.

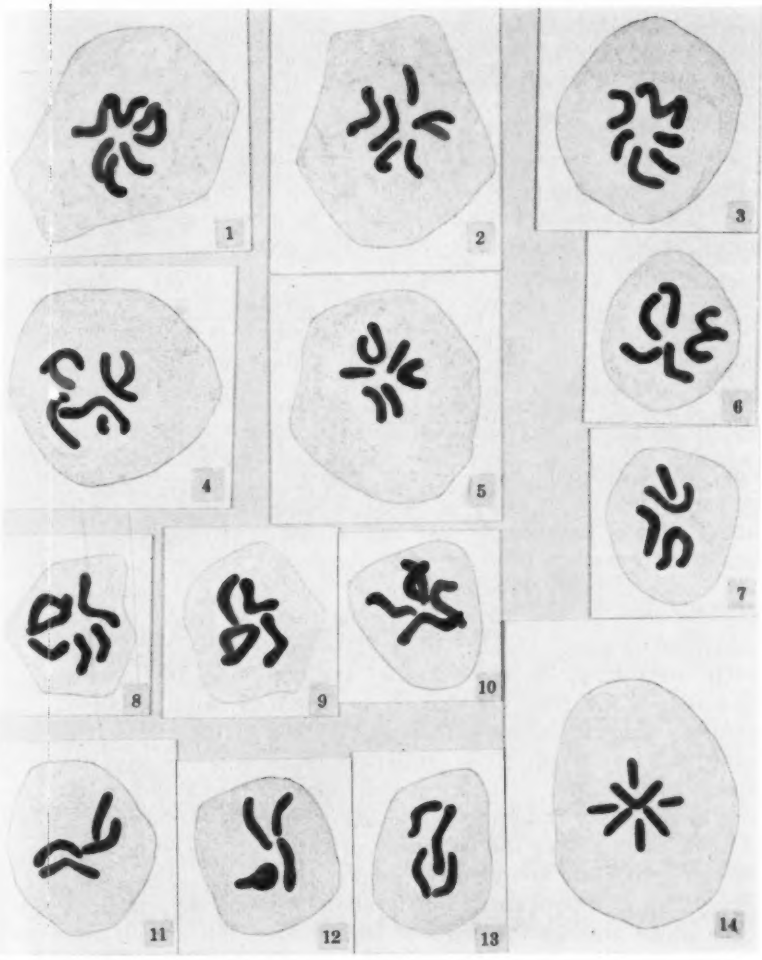

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Fig. 7: slide 2885-V; ovary; entire. In this ovary there are 5 mitotic metaphase figures in a cluster. Beside these are 2 cells which may be in prophase and may belong to the same cluster. The chromosome group, shown in Fig. 7, consists of not more than 5 members, 2 small V's and 3 rods. The other 4 mitotic figures are likewise obviously deficient, with not more than 5 chromosomes each, although the shapes and sizes cannot be determined accurately. Since they form a cluster, and no other mitotic figures are present in the gonad, these 5 are presumed to be alike and to have descended from a single ancestral cell. The deficient cells in this group do not appear to be much smaller than most of the other cells in the ovary. The cluster is near one edge of the ovary. The total number of cells in the gonad is difficult to estimate, but appears to be well over 100. Some of the interphase nuclei in the ovary are in a stage showing "prochromosomes" and apparently have 9 such bodies indicating that they have the normal, expected chromosome group.

Fig. 9: slide 2899-IV; ovary; sections. This case is similar to the one just considered (Figs. 7, slide 2885-V). It involves a cluster of deficient cells in an ovary sectioned longitudinally. The cluster lies about midway between the two ends of the relatively large, elongate gonad, and extends from one side about half way across the gonad. It extends through three or more sections, and is estimated to contain between 15 and 20 cells. Among these are eight in which the chromosome group consists of 5 or possibly 4 members. Being in early metaphase, the chromosomes are not lying flat in one plane and shapes and sizes are difficult to determine with precision. The evidence suggests strongly that all the cells in the cluster have the same deficient group. It is significant that the presence of the relatively large cluster of deficient cells here does not distort the outline of the ovary to form either a depression or a protuberance where it is in contact with the periphery of the ovary. The deficient cells just described are considerably smaller than many other cells in the ovary (all in interphase). But in addition there are numerous other cells which are like the deficient ones in size, but are in interphase. These are adjacent to the deficient mitotic figures, which suggests that they represent one or more additional clusters of deficient cells, in which mitosis is not synchronized with those described here.

Figs. 11, 12, 13: slide 2886-III; ovary; whole mount. Not only the

small number of chromosomes, but other features make this case especially interesting. The ovary is entire, not sectioned; there are two clusters of deficient cells in mitosis; both clusters appear to have the same chromosome group; and judging from its composition, this group apparently could be a haploid group, in which case it might be derived from a polar body instead of from the zygote. The two clusters of deficient cells lie toward, but not at, one end of the ovary; one of them is flattened somewhat against the peripheral membrane; the other is larger and, like most of the deficient clusters, either lies entirely away from the periphery or extends well away from it into the center of the ovary. In the larger cluster are 7 mitotic figures, approximately in metaphase, with 4 chromosomes each. Three of these groups are shown in figures 11, 12, and 13. The group consists of 1 small V and 3 rods. Although no figures in the other cluster are suitable for accurate analysis the chromosome number and general appearance seems to duplicate that in the larger cluster. Much, if not most, of this ovary may be haploid; but in one portion of it a few early prophase figures appear to have at least 9 chromosomes, including at least one large "limited" chromosome. No mitotic figures are found in the other ovary.

As might be expected, a few cases were found which appear to be comparable to those described above, but in which the evidence is not satisfactory because the cells might be cut or the figures are not clear. In one, the chromosome number appears to be 4 or 5, in another 3. The latter merits note because of the small number. The specimen (slide 2906-III) happens to be from one of a very few male larvae included in the study. The testis is sectioned and is very small. Apparently there are six mitotic figures in a cluster, each with 3 chromosomes—presumably 1 V and 2 rods. But no figure is both clear and in a cell that is clearly uncut. Hence the evidence is recorded without including a drawing. There is good ground for considering the cluster of cells (apparently about 8 in number) as being deficient in chromosome number, but the number may be more than three.

Supplementing the evidence from cells in the gonads, presented above, are two cases involving non-germinal material. These are described briefly here because each contributes somewhat to the subject. The first (slide 2918-IV) is from the series of preparations

dealt with above. In this slide a single cell in somatic tissue (possibly a neuroblast cell) exhibits a complete, flat metaphase plate with one of the V-shaped autosomes missing, as shown in figure 14. It should have 2 V's in addition to the 6 rods. Another somatic cell in the same preparation has the two V's as expected. Since somatic mitoses are rare in larvae at this stage, and only a small amount of somatic tissue was included with the gonads on this slide, no further data were secured for comparison in this case. The case seems to show that loss of one of the V-shaped autosomes does not act as a cell lethal (see discussion below).

The other supplementary case is that of an entire egg in the blastoderm stage (slide 2929-I). It is included because it involves aberrant behavior in a specimen in a pure wild-type stock (from Anaheim, California) not carrying the gene for "swollen" or any other mutant gene so far as known; and also because of the type of phenomena exhibited. In the blastoderm there are many mitotic figures in metaphase to late anaphase and apparently all, or nearly all, have the simple haploid somatic chromosome group of one V and three rods. In contrast, the germ line nuclei have 7 or 8 chromosomes. These are in a prochromosome stage, which prevents identification of individual types of chromosomes, such as the "limited" chromosome. The somatic group here resembles that found in the oocyte or a polar body nucleus following meiosis, except for the absence of any "limited" chromosome. This suggests that the soma may have been derived from such a haploid nucleus, without fertilization, and that the "limited" chromosome may have been eliminated in the normal manner during early cleavage. This view is supported by the presence of numerous chromatic bodies in the egg cytoplasm which could represent the eliminated "limited" chromosomes. Similarly, the constitution of the germ line nuclei resembles that of the sperm, suggesting that the germ line is developing without any maternal chromosomes. But the situation is far from clear. The chromatic bodies or clumps in the cytoplasm are of various sizes: many too large to represent one or two chromosomes; some large enough to represent several chromosomes. It is possible that fertilization occurred and that all paternal chromosomes were eliminated from some or many nuclei. There are possible resemblances to conditions previously described in Sciara species hybrids (Metz, 1959).

For the present the data are of interest mainly in giving further indication of the functional ability of deficient cells or nuclei during development and differentiation of soma as well as germ line.

Possible Derivation of the Deficient Chromosome Groups

Usually when deficiencies of whole chromosomes are observed they are attributed to non-disjunction in which the two daughter halves of a chromosome fail to separate and both go to one pole in mitosis, leaving the other pole deficient. But in Sciara, where certain chromosome eliminations occur normally, we should ask first whether these normal eliminations could be involved at all in the loss of chromosomes here. Although at best they could only be partly responsible, there are grounds for thinking that they do contribute to the end result. These reasons include the following:

(1) If non-disjunction were primarily responsible we would expect the process to give cells with extra chromosomes in proportion to the deficient ones, and we have not found such cells. (2) In *Sciara coprophila* the germ line and soma are set apart in the egg before the fifth cleavage division. At the fifth or sixth division of nuclei in the soma-line the large "limited" chromosomes are completely eliminated; but they are retained in the germ line (DuBois, 1933). Apparently none of the deficient chromosome groups dealt with here contains a "limited" chromosome. This suggests at once that the deficient cells may be descendants of somatic forerunners which, through aberrant behavior after elimination, became located in the germ cell region of the egg and subsequently became part of the gonads. In that case they should contain the normal female somatic group of eight chromosomes instead of the expected oogonial number of 9 or 10; and to this extent the deficient groups would owe their deficiency to aberrant cell behavior rather than chromosome behavior. Geyer-Duszynska (1959) has shown that incorporation of functional somatic nuclei into the germ line, after normal chromosome elimination, may be induced in Cecidomyids. (3) If, as the evidence seems to indicate, each cluster of deficient cells here is descended from a single deficient ancestral cell, the number in the cluster gives some clue as to how many cell divisions must have taken place during the multiplication process, at least while in the germ line. The numbers, as recorded above, support the view that probably in each case the initial chromosome loss

occurred before the gonads were formed. In that event it might have occurred in a somatic cell and this cell, or one or more of its descendants, might later have entered the germ cell region and in effect become part of the germ line.

If this hypothesis is correct it accounts for the loss of two chromosomes, the large "limited" chromosome and one of the original three X's (which normally is also removed during cleavage in eggs developing into females). Likewise it indicates that not only these but all the losses dealt with here occurred while the nuclei were in the soma region, before entry into the germ line. We may, therefore, be dealing with deficient somatic nuclei comparable to those found by DuBois (1933) in late cleavage. Those deficient groups, like the ones we have found, range down to four and three chromosomes — the latter less than half the number expected even after allowing for normal eliminations. Obviously, radical and abnormal events are involved. We have no direct evidence as to what they are. Since an adequate discussion of all the possibilities would be too long to be justified here we will only make the following suggestions: (1) The deficient groups are probably not all brought about in the same manner. (2) Some, at least, of those with four chromosomes are probably haploid groups, derived from polar body nuclei which induced cell formation and developed parthenogenetically, as polar body nuclei have been found to do (See Stalker, 1956 Murdy and Carson, 1959, Sprackling, 1960). (3) In some of the other deficient cell clusters the chromosomes may well have been derived from the aggregates of chromosomes produced by division of polar body chromosomes before or during early cleavage stages. In Sciara, many such chromosomes are often found loose in the cytoplasm of the egg, and sometimes close to cleavage nuclei where they might be induced to form nuclei and to undergo development. (4) In still other cases, especially where five or more chromosomes are present, the deficiency may be due to loss from nuclei having the normal expected group. Possibly, through some abnormal circumstance, the influence which normally induces the elimination of a "limited" chromosome or an X may have acted similarly on one or more other chromosomes, to bring about their removal. DuBois (1933) postulated that occasionally an ordinary chromosome might become entangled with a large "limited" chromosome at the time of elimination and be lost with it. It is hard to see how this process

could result in such extensive reduction in chromosome number as that observed, however, without the presence of some environmental or genetic factor to increase greatly what might be considered a spontaneous or chance frequency. Indeed, this same problem arises in connection with any or all of the suggestions just set forth and serves, in turn, to raise the question as to whether the mutant gene for "swollen" or some other genetic element in our material may be such a factor (see discussion below).

The last mentioned points become especially cogent when we consider the fact that the deficient cell clusters described here are only those in which the cells are at approximately the metaphase of mitosis and that the actual number of deficient clusters should be several times as great. It seems not unreasonable to assume that in the original lot of material at least five or six times as many cases of deficient groups are present as have been detected.

Morphogenetic Aspects

At this point a few notes may be recorded concerning morphogenetic features. In the deficient cells themselves, as already indicated, we have detected no differences from normal except in size and in chromosome number. In cell grouping the deficient cells appear to behave normally; they form clusters, as do ordinary oogonia, within each of which mitoses occur synchronously and independently of other cell clusters in the gonad. Likewise, these deficient cell clusters apparently contribute or respond normally to morphogenetic influences, and take part in the formation of an ovary which is normal in shape and contour. As the normal Sciara ovary develops during the stages dealt with here it changes from a short oval (Berry, 1941) to an elongate oval of characteristic shape. In the ovaries under consideration we have detected no indication of abnormality either in general form or in local contour such as bulging or depression of the surface at or near the deficient cluster.

Consideration of Individual Chromosomes

When one considers the deficient chromosome groups in relation to the viability and functional activity of the cell, attention is drawn immediately to the individual chromosomes, or chromosome pairs. It seems significant, for example, that the large "limited" chromosome

appears to be absent in all cases, although normally it should always be present in the oogonia. At the opposite extreme, both members of the pair of autosomal V-shaped chromosomes may be present in all the deficient oogonia with more than four chromosomes, and one member is present in the oogonia with four, and possibly three, chromosomes. This suggests that the V may be essential for cell viability here. In the case of the rod-like chromosomes, individual pairs cannot be distinguished, but certain deductions may be made. For example, in a group of five chromosomes which contains the pair of V's, the three rods must include one member of each pair, or else both members of one pair and a single member of another pair, in which case the third pair is entirely absent. When the chromosome group consists of one V and three rods it may represent a haploid group derived from a polar body, as explained elsewhere, rather than extreme loss of chromosomes. Despite the small number of chromosomes in the latter case the balance between the different kinds would be maintained. In the groups with five or six chromosomes, however, there must be considerable asymmetry or else complete loss of at least one pair.

Discussion

As already intimated, the limitations of the present data are such that a lengthy discussion would not be justified. Most of what seems to be needed has been included above under the respective headings. The remainder is set forth briefly below.

Concerning the basic problem of chromosome function in relation to development and differentiation, the present evidence indicates that in the Sciara germ line, cells with radically reduced and unbalanced chromosome groups are able to continue functioning in an apparently normal manner through the stage of larval development dealt with here. This includes not only continued growth and reproduction of the cells but migration, along with the other germ cells, from the apical germ cell region in the egg to the definitive site of the future gonads in the developing embryo, and also taking part in the formation and growth of early larval gonads of normal appearance. Interest here attaches primarily, of course, to the deficient cells which are not haploid. The haploid ones might be expected to function normally under these conditions, if they developed at all (see e.g., MacKnight, 1937).

It should be recalled, however, that little if any real differentiation occurs in these processes, other than what may be involved in the formation of the thin enveloping membrane and in the determination of the shape of the gonad. The question remains as to whether, or how far, the deficient cells could continue to function in the later stages involving follicle formation, differentiation of nurse cells and oocytes, and meiosis. Few studies have been made on these later stages in the ovary, but our extensive studies on comparable stages in the testis, including meiosis — studies ranging over numerous species of Sciara — have not revealed conditions like those treated here.

Concerning the frequency and cause of the deficient chromosome groups little more can be said at present except that the frequency seems very high to be the result of purely fortuitous events. If we assume that the events are not fortuitous two additional speculations merit attention. (a) Aside from the possibility noted above, that a genetic factor in the "swollen" or Anaheim stocks is responsible, a different type of causal agent might be postulated, namely one produced in overripe eggs which result from delayed fertilization. It might be that in the studies considered here females were kept longer than usual before being bred and abnormal chromosome behavior was induced as a consequence of overripening. Overripening may take place on a large scale in Sciara because all the eggs in a female ripen at once and are normally retained until mating occurs. If mating is delayed they may all become overripe. Conceivably conditions produced by overripening might lead to the induction of abnormalities in the germ line either directly or through initial effects on somatic nuclei or polar body derivatives which later behaved abnormally and became incorporated into the germ line. (b) If the gene for "swollen" should be involved, the fact that half the female larvae in the present studies are homozygous and half heterozygous for this gene is important. Deficient cells might only be produced in the homozygous individuals. In that case, judging by the estimated frequency rate noted above, all such individuals might well possess deficient cell clusters.

Although somewhat outside the scope of the present paper, one further point calls for brief comment. As noted above, it seems probable from the evidence that the chromosome losses dealt with here occurred in the somatic region during cleavage mitoses. If that

is not the case, and losses such as these can take place in the germ line, it would be important to find out *how* they occur, because of the light this might throw on the general problem of selective chromosome behavior and the mechanism of mitosis. The only type of chromosome elimination known in the Sciara germ line involves movement of a condensed X chromosome directly through the nuclear membrane into the cytoplasm during interphase (Berry, 1941). Could the "limited" chromosome and some of the autosomes be extruded in the same manner or would they be lost during mitosis as in the soma? And in either case what is responsible for the behavior? Suitable studies on Sciara might provide significant information.

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